Phosphodiesterase-4-selective inhibitors have therapeutic potential for treating major diseases such as asthma and chronic obstructive pulmonary disease, as well as depression, Parkinson's disease and Alzheimer's disease

Keynote review:

Phosphodiesterase-4 as a therapeutic target

Miles D. Houslay, Peter Schafer and Kam Y.J. Zhang

Cyclic AMP (cAMP) is a key second messenger in all cells. It is compartmentalized within cells and its levels are controlled, as a result of spatially discrete signaling cassettes controlling its generation, detection and degradation. Underpinning compartmentalized cAMP signaling are ~20 members of the phosphodiesterase-4 (PDE4) family. The selective inhibition of this family generates profound, functional effects and PDE4 inhibitors are currently under development to provide potential, novel therapeutics for the treatment of inflammatory diseases, such as asthma, chronic obstructive pulmonary disease and psoriasis, as well as treating depression and serving as cognitive enhancers. Here, we delineate the range of PDE4 isoforms, their role in signaling, their structural biology and related preclinical and clinical pharmacology.

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Functional significance of phosphodiesterase-4 isoforms

Cyclic nucleotide phosphodiesterases (PDEs) provide the only route for degrading cyclic AMP (cAMP), a key second messenger inside cells, thus providing a pivotal means of regulating cAMP levels. Their importance is emphasized by the multiplicity of cAMP-hydrolyzing PDEs, encoded by the human genome, and their high conservation between species and during the selective pressures of evolution. Of these, the multi-gene phosphodiesterase-4 (PDE4) family has attracted considerable attention over the past decade because PDE4-selective inhibitors have potential therapeutic use in a range of major disease areas. During this time the development of increasingly efficacious inhibitors has progressed alongside a dramatically enhanced understanding of PDE4 biology. In this review we will discuss the cellular and molecular biology of PDE4 isoforms (of which ~20 have been characterized) and, in particular, the pivotal role that PDE4 isoforms play in achieving compartmentalized signaling in cells by interacting with various scaffold proteins. We will review the dramatic advances that have recently been made in our

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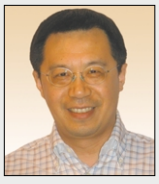

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appreciation of the structural properties of the PDE4 catalytic unit and its implications for inhibitor development. Finally, we will review the current biology that underpins the therapeutic potential of PDE4 inhibitor action.

Anchored PDEs underpin compartmentalized cAMP signaling in cells

Signaling systems control and coordinate all aspects of cellular function, providing key targets for drug discovery. A major challenge in the initial stages of drug discovery, targeted at cellular signaling systems, is to identify appropriate control points and to generate selectivity for the target protein.

In many instances isoenzymes are present at key control points in signaling networks, providing feedback regulation and cross-talk between different signaling pathways that are tailored on a cell type-specific basis. An example of this is seen with Raf isoforms that serve to control activation of the ERK signaling pathway and where expression of c-Raf confers inhibition by cAMP, whereas expression of B-Raf confers activation by cAMP [1].

cAMP provided the paradigm not only for the intracellular messenger concept but also for signal compartmentalization within cells [2,3]. The basic signaling cassette is, in this instance, elaborated upon by a series of isoforms. This allows different cell types to specify distinct response modes for their cAMP signaling systems in terms of spatial compartmentalization, sensitivity and their consequential action. The cAMP signaling cassette features distinct G-protein coupled receptors, isoforms of adenylyl cyclase, isoforms of protein kinase A (PKA), a broad family of PKA anchor proteins (AKAPs), two cAMP-stimulated GTP exchange proteins (EPACs) and a large family of cAMP-hydrolyzing PDEs that provide the only route for cAMP degradation in cells [2–7].

There are eight PDE families capable of hydrolyzing cAMP. Some hydrolyze cAMP exclusively, whereas others hydrolyze cAMP and cGMP [2]. The provision of point sources of cAMP generation within defined subdomains of the cell surface plasma membrane, coupled with tethered PDEs, generates spatially distinct intracellular gradients of cAMP. These gradients can be detected in living cells by various fluorescence energy resonance transfer (FRET) probes [8]. They enable the selective activation of appropriately anchored PKA subpopulations with associated target substrates [3,7]. PDEs provide the basis for generating compartmentalized cAMP signaling [6,8] and this has been most extensively studied for the PDE3 and PDE4 families. Recent studies on cardiac myocytes show that these enzymes are localized at different sites within the myocytes, generating distinct effects on intracellular gradients of cAMP [9].

PDE4 activity is provided by a large and diverse family of isoforms

PDE4 enzymes specifically hydrolyze cAMP, they are widely expressed and they play major regulatory roles, as deduced from the action of highly selective inhibitors [10], targeted gene knockout [11–15], small inhibitory RNA (siRNA) ablation [16] and dominant–negative-mediated disruption of enzyme intracellular targeting [17]. They are found in many cell types and tissues including leukocytes, airway and vascular smooth muscle, vascular endothelium and brain. The involvement of PDE4 in pathological processes associated with these tissues suggests a great potential for pharmacological intervention in a variety of inflammatory, vascular, angiogenic and neurological disorders. Through modulation of cAMP levels, PDE4 regulates leukocyte responses including the pro-inflammatory actions of monocytes, T cells and neutrophils, airway and vascular smooth muscle constriction, and neurotransmitter signaling through adenylyl cyclase-linked G-protein coupled receptors [such as that for N-methyl-D-aspartate (NMDA)]. Potential diseases for PDE4 inhibitor therapy include asthma, allergic rhinitis, atopic dermatitis, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, psoriasis, Crohn's disease, cancer, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, schizophrenia and depression [10,18–22].

Many PDE4 isoforms, of which ~20 are known to date, provide a considerable challenge to understanding the range of actions of this family. However, through selective expression, this diversity offers the ability for tailoring cAMP signaling on a cell type-specific basis because PDE4 isoforms exhibit distinct targeting and regulatory properties [5,6]. The fact that such a large and complex array of enzymes is highly conserved points towards the importance of the PDE4 family.

Individual isoforms generated by the four PDE4 families (A, B, C and D) are each characterized by unique N-terminal regions (Figure 1). These families are believed to play a major role in conferring isoform-specific targeting to distinct intracellular sites and signaling complexes [6,23], thereby underpinning compartmentalization of cAMP signaling. The use of distinct promoters for isoforms [24–27] together with control of mRNA stability [28] allows cells to exhibit specific patterns of PDE4 isoform expression.

PDE4 isoforms are divided into three groups (Figure 1) [6]. Long isoforms have two regulatory domains, called upstream conserved region 1 (UCR1) and upstream conserved region 2 (UCR2), located between the isoform-specific N-terminal region and the catalytic unit. Short isoforms lack UCR1 and super-short isoforms lack UCR1 and have a truncated UCR2. A key functional role of the UCR modules is in determining PDE4 regulation through PKA and ERK phosphorylation [5,6]. UCR1 contains a PKA phosphorylation site, allowing long isoforms to be activated by this enzyme [29,30], thereby providing an important part of the cellular desensitization machinery for cAMP signaling by increasing the cellular capacity for cAMP degradation [30,31]. The catalytic unit of all isoforms, except those from the PDE4A subfamily, can be phosphorylated by ERK [32–34], a kinase that is activated by pro-inflammatory

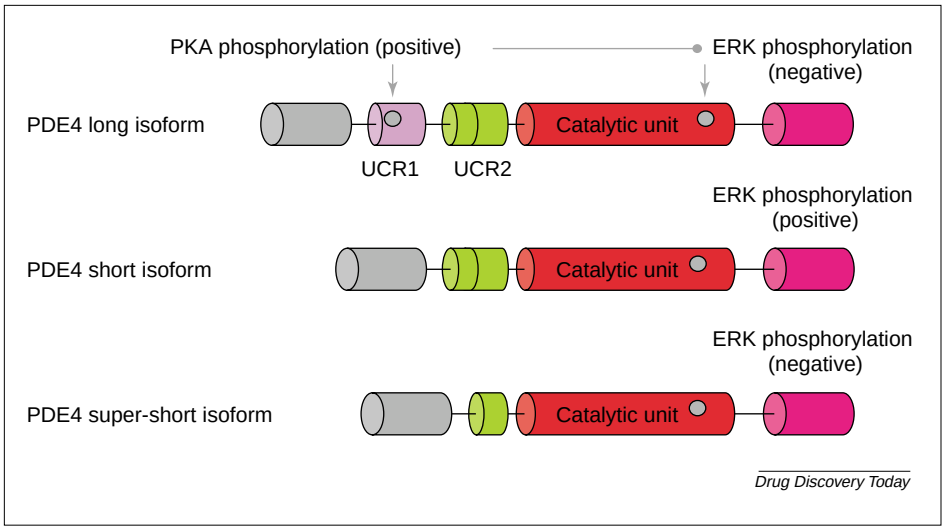

FIGURE 1

PDE4 isoforms. The three groups of PDE4 isoforms are shown here, they are defined by their unique N-terminal region. The regulatory UCR1 and UCR2 regions are indicated together with catalytic unit and sites for phosphorylation by PKA (on UCR1) and ERK (on the catalytic unit) are indicated. The isoform-specific N-terminal region is shown in grey and the subfamily-specific C-terminal region is shown in pink.

stimuli [35] and plays a role in memory processes [36]. The UCR modules orchestrate the functional outcome of ERK phosphorylation. Inhibition is seen in long isoforms, activation in short isoforms and no effect is detected on super-short isoforms [34]. Changes in the relative levels of long isoforms compared with short isoforms radically affects the consequences of ERK activation on cAMP signaling. This is highlighted in monocyte to macrophage differentiation; long PDE4D isoforms predominate in monocytes whereas short PDE4B predominates in macrophages [35]. Thus, pro-inflammatory mediators trigger an overall decrease in PDE4 activity in monocytes but they elicit an overall increase in PDE4 activity in macrophages [35].

Inhibition of PDE4 long isoforms by ERK phosphorylation is transient because an inbuilt feedback regulation is provided by the ensuing increase in cAMP, causing PKA to phosphorylate UCR1, thereby overcoming inhibitory ERK phosphorylation [33]. On this basis ERK activation can elicit a transient rise in cAMP as a result of its action on PDE4 long isoforms. The ability of PDE4 isoforms to bind ERK at specific docking sites, and to respond in very different ways to phosphorylation by ERK, clearly exemplifies the importance of PDE4 isoforms in transducing the actions of ERK on cAMP signaling.

In addition to their regulatory roles, UCR1 and UCR2 provide interfaces for interaction with the scaffolding protein myomegalin, the immunophilin XAP2 and phosphatidic acid [5,37,38]. Furthermore, UCR1 and UCR2 appear capable of interacting with each other. This can occur internally within a particular PDE4 molecule [39] but it could also offer a means of facilitating homodimer formation in long isoforms [40]. In every instance it appears that the interactions between UCR1 and UCR2 generate conformational changes in the PDE4 catalytic unit, which are linked to activity changes seen upon phosphorylation by PKA and ERK and to consequential responses upon the binding of certain inhibitors, such as rolipram. Indeed, the fact that certain PDE4 inhibitors appear to deviate from Michaelis–Menten kinetics might be explained by actions on dimeric forms of PDE4, where the binding of an inhibitor to one component of the dimer affects the binding of an inhibitor to its partner.

Inhibitors and conformational switching in PDE4

It is believed that the PDE4 catalytic unit from all four subfamilies can adopt conformationally distinct states. This idea was formed as a result of the different sensitivities of PDE4 preparations to inhibition by rolipram, the discrimination between the *R* and *S* rolipram enantiomers and binding studies with [^{3}H]-rolipram, which identified low- and high-affinity components [41]. For a long time, there has been considerable interest in this subject as it was originally suggested that PDE4 inhibitors that are able to interact with the so-called high-affinity rolipram binding sites (HARBS) and the low-affinity rolipram binding sites (LARBS), PDE4 conformers, are more likely to cause emesis as a side-effect than those that only interact with LARBS. However, such a hypothesis has unfortunately proved to be too simplistic and is no longer exploited in compound development.

The molecular basis of the conformational switching of the PDE4 catalytic unit *in vivo* is poorly understood but several *in vitro* model systems have shown how these changes can occur. One model used recombinant PDE4A to show that alterations in rolipram binding and alterations to its enantiomers could ensue, depending on whether the enzyme had bound Mg^{2+} in its active site [42,43]. In this model system, using purified recombinant enzymes expressed in *Escherichia coli*, it was demonstrated that affinity for rolipram interaction was considerably lower in the apoenzyme compared with the Mg^{2+}-bound holoenzyme. However, under physiological conditions the concentration of intracellular Mg^{2+} is far in excess of the concentration needed to saturate the binding site on PDE4 [30]. This makes it extremely unlikely that significant levels of PDE4 apoenzyme occur under physiological conditions. Thus, there must be mechanisms whereby active Mg^{2+}-bound holoenzyme can be triggered to switch its affinity for rolipram between HARBS and LARBS states. Furthermore, there is a lot of evidence that active forms of PDE4, showing either low or high affinities for rolipram inhibition, occur (e.g. PDE4A) [44–46] and that various crystal structures of a catalytically active, low-affinity enzyme (with bound Mg^{2+}) can be generated. By contrast

TABLE 1

PDE4 interacting proteins[a]

Interacting protein	PDE4 isoform	Comments on interaction	Rolipram inhibition
SH3-domain-containing proteins	PDE4A4, PDE4A5, PDE4D4	Shows specificity for SH3 domain (e.g SRC family tyrosyl kinases). All human PDE4A species interact weakly with SH3 domains via LR2 region.	Increases sensitivity of PDE4A4 to inhibition by rolipram.
RACK1	PDE4D5	Binds to region in unique N-terminal region.	Decrease in sensitivity to inhibition by rolipram.
Myomegalin	PDE4D3	Shown for PDE4D3. Interacts with UCR2, implying all isoforms can bind.	Unknown.
β-arrestin	PDE4D5; all PDE4	Conserved interaction site within catalytic unit allows all PDE4s to interact. PDE4D5 has additional interaction site within its unique N-terminal region.	None noted for isoforms examined.
XAP2 (AIP, ARA9)	PDE4A4, PDE4A5	Binds to unique N-terminal region of indicated PDE4A isoforms and also binds to a site in UCR2.	Increases sensitivity to inhibition by rolipram.
mAKAP	PDE4D3	Involves unique N-terminal region. PKA phosphorylation of Ser13 in N-terminus of PDE4D3 enhances interaction.	Unknown.
AKAP450	PDE4D3	Involves unique N-terminal region.	Unknown.
ERK	All	Interacts with docking and specificity sites in the catalytic unit that straddle the phosphorylation site for ERK found in PDE4B, PDE4C, PDE4D but not PDE4A isoforms.	None noted for isoforms examined.

[a]References [8,29,34,37,38,46,51,58,60–63,65,118,119].

(Mg^{2+}-free) apoenzymes, with low-affinity states for rolipram-binding [42,43], will be catalytically inactive. Nevertheless, such studies on Mg^{2+} action [30,42,43,47] raise the intriguing possibility that changes in the orientation of residues involved in coordinating Mg^{2+} in the PDE4 catalytic unit could provide a means for triggering an overall conformational change in the PDE4 catalytic site, which has been exploited in analyses of PDE4A4 foci formation. In this regard, phosphorylation of PDE4 long isoforms by PKA has been shown to cause a small increase in their affinity for Mg^{2+} and rolipram [30,48]. To date, crystallography studies done with PDE4 have focused on the isolated catalytic unit because of the propensity of purified, full-length enzymes to aggregate at high concentrations. The structures obtained come from various PDE4 families and many investigators have revealed almost identical structures. These reflect an active form of PDE4 that has low (μM) affinity for rolipram. Such observations are consistent with the contention that portions located N-terminally to the PDE4 catalytic unit are needed to trigger the conformational change that results in altered sensitivity to inhibition by rolipram [49]. Indeed, it is in such regions that PDE4 interacts with various other proteins and is phosphorylated by PKA. It seems probable that the conformational transition triggering altered sensitivity to rolipram can be achieved via several different routes, which seem likely to be mediated by actions that involve the Mg^{2+}-binding site [42,43]. Several examples of triggers have been identified including one that is specific to the PDE4A4 isoform occurring when it binds to SH3 domains of SRC family tyrosyl kinases [46]. This provides a paradigm for protein–protein interaction affecting rolipram inhibition (Table 1). Further evidence that protein–protein interaction can affect inhibition by rolipram is evident in the association of PDE4A5 with XAP2 [37] and PDE4D5 with RACK1 [50,51]. The ability of PKA phosphorylation of PDE4D3 to increase sensitivity to rolipram inhibition [47] appears to ensue through a conformational change that is distinct from the one that causes activation [48] and could relate to changes in either internal UCR1–UCR2 interaction [39] or homodimerization [40]. Indeed, dimerization of PDE4 at a surface that involves the UCR1–UCR2 interface [40], rather than just the catalytic unit as exemplified in various crystal structures [52], could provide an important means of regulating the conformation of the PDE4 catalytic site and its function. It is now unequivocally established that there is a single rolipram-binding site on PDE4, within the catalytic site [53–55], but the affinity for rolipram can be changed by the action of appropriate switches.

The possibility of developing subfamily-selective inhibitors poses considerable challenges, although generating inhibitors with reasonable selectivity for PDE4D has been recorded [56]. In attempting to screen for PDE4 subfamily-selective inhibitors, great care must be taken when setting up assays so that results are not inadvertently biased. This can occur if a panel of PDE4 isoforms in different intrinsic conformational states is used, because of the presence of interacting proteins and differences in phosphorylation. If some PDE isoforms used in the assay are modified but others are not then aberrant indications of selectivity might occur. As a general rule the set of enzymes reflecting each PDE4 subfamily should exhibit similar sensitivity to inhibition by rolipram. Rolipram IC_{50} values in the 500 nM to 2 μM range (with 1μM cAMP substrate) are indicative of low-affinity conformers, whereas IC_{50} values in the 10 nM to 100 nM range, such as that generated with human PDE4A4-SH3 domain interaction [44,46], are indicative of a high-affinity conformation. If the panel of PDE4 enzymes from different subfamilies have vastly different

affinities for rolipram to begin with, as is evident in various studies, then it is inevitable that certain compounds will seemingly appear to be selective; however this is simply because the panel reflects enzymes that are in different conformational states.

It is also prudent to determine the action of compounds on PDE4 enzymes in cells that express the therapeutic target, as cell type-specific interacting proteins and phosphorylation might affect PDE4 sensitivity to inhibitors. In such instances the PDE4 activity of each subfamily can be analyzed after selective immunopurification [45].

The non-phosphorylated PDE4B and PDE4D catalytic units examined in all crystallography studies done to date reflect PDE4 in a LARBS state. The challenge for the future will be to obtain structures for full-length molecules alone as well as those complexed to other proteins. This will help scientists to understand the changes occurring in the active site that lead to HARBS. This is a formidable quest as full-length species show a profound propensity to aggregate upon purification.

Protein–protein interaction characterizes the PDE4 family

Each PDE4 isoform is characterized by a unique N-terminal region. The notion that this was involved in intracellular targeting originated from studies on PDE4A1, the simple deletion of the N-terminal region converted an entirely membrane-associated enzyme into a fully active cytosolic one [23]. Subsequent studies showed that the membrane association of PDE4A1 was conferred by the Ca^{2+}-triggered insertion of its N-terminal TAPAS-1 domain into the lipid bilayer [57]. Such lipid interaction is unusual in PDE4 isoforms as intracellular targeting and assembly, into signaling complexes, is invariably determined through protein–protein interactions [5,6]. A growing number of PDE4 protein partners have now been identified (Table 1). Several of these species are signaling scaffold proteins, such as β-arrestin [8,58,59], AKAPs [60,61] and RACK1 [51]. Recruitment of PDE4 to such modules allows localized gradients of cAMP to be generated. This has a controlling action on tethered effectors of cAMP activity, such as PKA, and provides the basis of compartmentalized cAMP signaling. Indeed, AKAP-recruited PDE4 long isoforms provide a self-regulatory module [61], a method of gating the activation of anchored PKA [17] and a way of preventing PKA from being inappropriately activated by fluctuations in basal cAMP levels [17]. Thus, when cAMP levels rise sufficiently to activate PKA this serves to phosphorylate and activate PDE4, thereby lowering cAMP levels and de-activating AKAP-anchored PKA. This is a similar notion to that of the feedback interaction involving phosphorylation of PDE4 long isoforms by ERK [33].

Interaction with scaffold and/or anchor proteins directs the intracellular targeting of tethered PDE4 isoforms. Thus, the induction of mAKAP, occurring in cardiac myocytes upon hypertrophy, causes PDE4D3 to relocalize around the nucleus with mAKAP [61]. The intracellular targeting of PDE4A5 is disrupted in apoptosis as a result of caspase-3 activity. Caspase-3 cleaves within the unique N-terminal region of PDE4D5, removing the site where interactions with SH3 domains of SRC family tyrosyl kinases occur [62,63]. This action is thought to facilitate apoptosis in fibroblasts.

A functional example of dynamic redistribution of PDE4 in cells is provided by the PDE4–β-arrestin complex [59]. Thus, agonist occupancy of the β_2 adrenergic receptor (β_2AR) causes the transient recruitment of β-arrestin–PDE4D5 to the β_2AR, thereby recruiting an active PDE to the site of cAMP synthesis at the plasma membrane. The key functional role of β-arrestin-recruited PDE4D5 is to downregulate the phosphorylation of the β_2AR by AKAP79-tethered PKA, thereby attenuating the switching of β_2AR coupling from G_s-stimulation of adenylyl cyclase to G_i-mediated activation of ERK [16,58]. Such a system has been identified as being functional in cardiac myocytes [58,64].

Approaches using siRNA knockdown [16] and dominant–negative (engineered catalytically inactive) [58] PDE4 isoforms demonstrate that PDE4D5 preferentially associates with β-arrestin and regulates the phosphorylation of the β_2AR by PKA. Such selectivity is partly caused by the additional β-arrestin interaction site, found within the unique N-terminal region of PDE4D5 [65]. It is also because various other PDE4 isoforms are sequestered to interact with other anchors in cells [16].

It has recently been shown that co-ligation of the T cell receptor (TCR) with CD28 triggers the recruitment of a β-arrestin–PDE4 complex to lipid rafts [66]. The function of this is to remove the inhibitory action of PKA, mediated by Csk. This undoubtedly underpins the reason why PDE4 inhibitors are so effective in attenuating T cell activation.

Binary cAMP-independent action of PDE4-selective inhibitors

Phosphorylation and binding to other proteins trigger conformational changes in some PDE4 isoforms that modify sensitivity to various active-site-directed inhibitors. It is possible that the binding of some inhibitors can trigger a conformational change in certain PDE4s that could, conversely, affect their interaction with other proteins. In doing so, such compounds can elicit functional responses that are independent of cAMP. The paradigm for such an action has come from studies on PDE4A4 [67]. Thus, chronic exposure of cells to rolipram induces the redistribution of PDE4A4 to foci. This is evident using wild-type PDE4A4 but is most easily quantified in living cells using green fluorescent protein (GFP) -tagged PDE4A4. Redistribution does not start for several hours, is absolutely dependent upon protein synthesis and is independent of cAMP elevation. Indeed, gross elevation of cAMP ablates redistribution, presumably by displacing rolipram from the PDE4A4 active site. As with HARBS and/or LARBS, rolipram enantiomers have a discriminative effect with *R*(–) rolipram causing foci formation, whereas *S*(+) rolipram hardly has an effect. Surprisingly, only some PDE4 inhibitors elicit this effect

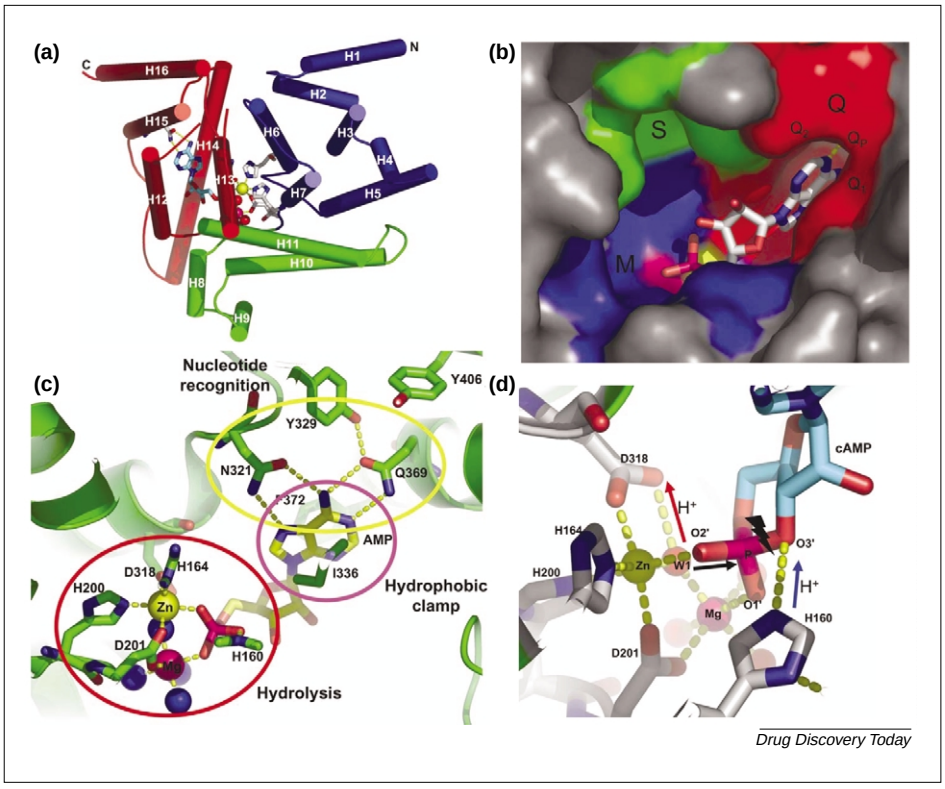

FIGURE 2

PDE4 structure, substrate and inhibitor binding to the active site and mechanism of hydrolysis. (a) The overall structure of the catalytic domain of PDE4D. The α-helices are represented by cylinders and the loops are represented by tubes. The α-helices labeled 1–16 are divided into three subdomains colored blue, green and red. The Zn^{2+} and Mg^{2+} are represented by yellow and magenta spheres, respectively. Residues coordinating the metal ions as well as bound AMP are shown as stick models. **(b)** A surface rendition of the PDE4D active site is shown with bound AMP as a stick model. The active site is divided into three subpockets: Q (red), S (green) and M (blue). The Q subpocket can be further divided into Q_1, Q_2 and Q_P regions (these are labeled). The yellow and magenta surfaces are Zn^{2+} and Mg^{2+} ions, respectively. **(c)** Residues in the active site can be clustered into three functional groups that are responsible for: nucleotide recognition; hydrophobic clamp; hydrolysis. These are highlighted on the co-crystal structure of PDE4D bound to AMP. **(d)** The mechanism of cyclic nucleotide hydrolysis is illustrated on the modeled cAMP bound to PDE4D. D318 deprotonates the bridging water W1 to generate a nucleophile that attacks the phosphorous (P) inline to the scissile bond. The leaving O3′ is protonated by H160.

indicating that the trigger for this action is a specific subdomain in the catalytic site, only probed by some compounds. This is consistent with the observation that certain point mutations made within the catalytic site sustain inhibition by rolipram but they ablate foci formation.

Foci formation provides a robust and intriguing phenomenon that supports the notion that conformational changes linked to the active site occur in PDE4 enzymes. Analysis of a wider range of inhibitors and understanding the formation of this complex is needed to generate SAR. Foci formation in PDE4A4 appears to be a binary phenomenon controlled by selective inhibitors. It can be used as an additional tool to subclassify PDE4-selective inhibitors and it is anticipated that, in the future, it can be linked to functional significance.

PDE4 structural biology

Catalytic domain structure

The crystal structure of the apo catalytic domain of PDE4B [68] reveals a compact α-helical structure consisting of 16 helices divided into three subdomains (Figure 2a). The active site forms a deep pocket located at the junction of the three subdomains and is lined with highly conserved residues (Figure 2b). A binuclear metal ion center, where Zn^{2+} is coordinated by conserved, paired histidines and aspartates and two water molecules, is found at the wider side of the active site. Mg^{2+} is also located there, coordinated by the same aspartate that coordinates Zn^{2+} as well as five water molecules (one of which bridges Mg^{2+} and Zn^{2+}). This minimal interaction with protein residues indicates that Mg^{2+} is not essential for structural integrity. Indeed, it would seem that the Mg^{2+} can be stripped off by chelators such as EDTA without affecting the proper folding of PDE4 [42,43].

The active site pocket contains 12 of the 17 conserved residues from the 21 PDE gene family members. Within the active site are three clusters of residues (Figure 2c) that underpin three factors: nucleotide recognition, where a cluster of residues determines the orientation of the amide group of an invariant glutamine for selective binding to either cAMP or cGMP; a hydrophobic clamp, where two highly conserved hydrophobic residues sandwich the planar purine ring involved in substrate binding; and hydrolysis, where a cluster of conserved residues near the di-metal center are responsible for cyclic nucleotide hydrolysis [69].

The active site can be subdivided into three pockets (Figure 2b) [54]: a metal-binding pocket (M-pocket), a solvent-filled side pocket (S-pocket) and a pocket containing the purine-selective glutamine and the hydrophobic clamp (Q-pocket). The M-pocket contains the di-metal ions and highly conserved hydrophobic, polar residues that coordinate the metal ions. The S-pocket mainly consists of hydrophilic amino acids and is filled with a network of water molecules in most of the inhibitor complexes. The Q-pocket can be subdivided into three distinct areas: a 'saddle' formed by the conserved glutamine and the P-clamp (Q_P) and two narrow, but deep, hydrophobic pockets (Q_1 and Q_2) that flank Q_P.

A 'glutamine switch' mechanism for nucleotide selectivity

In the PDE superfamily the amide group of an invariant glutamine in the active site adopts one orientation to interact with cAMP and switches to another orientation, by flipping 180°, to interact with cGMP. This so-called 'glutamine switch' [69] is controlled by an intricate network

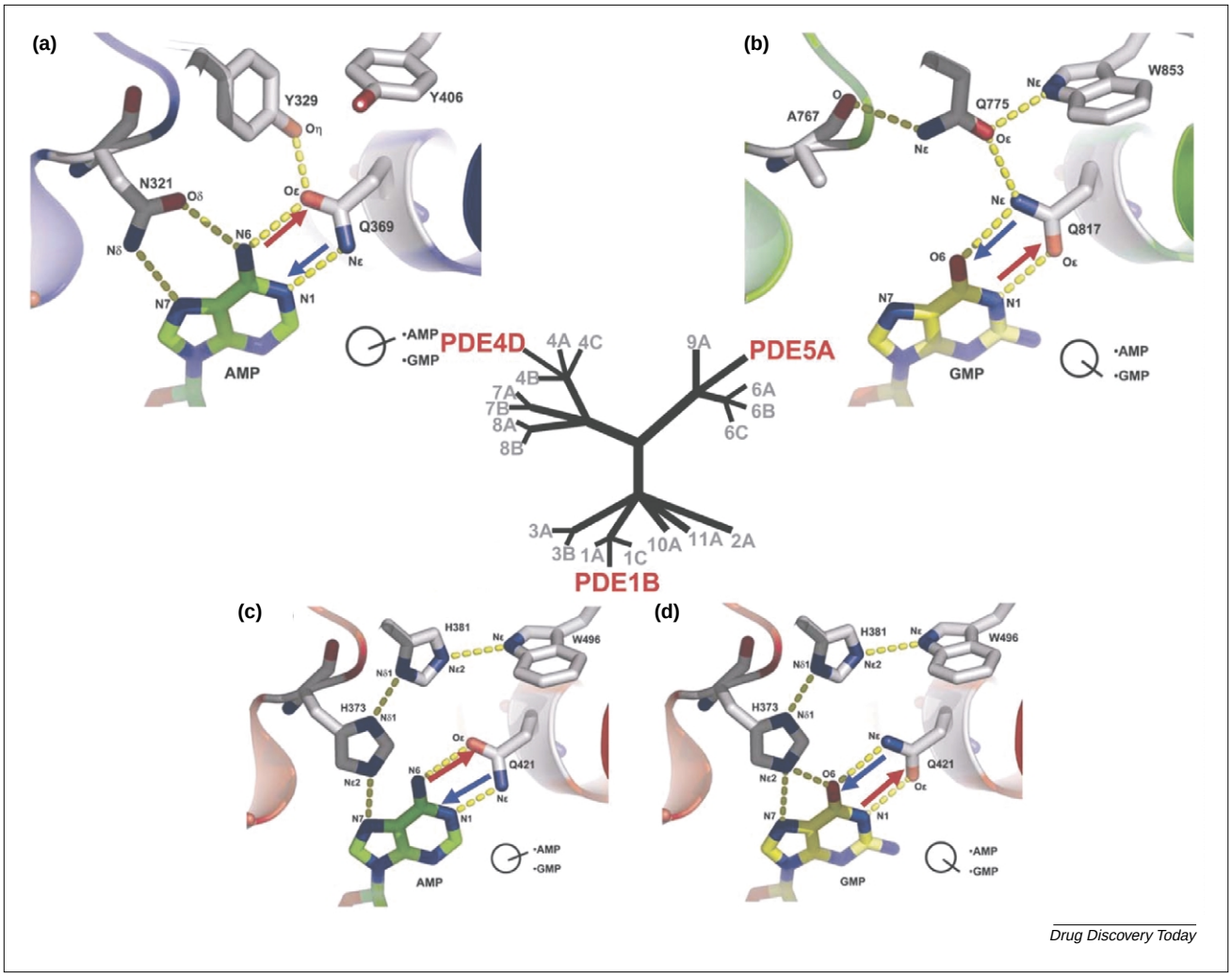

FIGURE 3

A glutamine switch mechanism of nucleotide selectivity. (a) Q369 recognizes AMP in PDE4D by forming a bidentate H-bond with adenine. Specifically, the Nε atom of Q369 donates an H-bond to the N1 atom of the adenine ring and the Oε accepts an H-bond from N6 in the exocyclic amino group of adenine. This particular orientation of Q369 is stabilized by H-bonding of Oε to the phenolic hydroxyl Oη of Y329. In addition, N321 forms a bidentate H-bond with the adenine base by donating one H-bond from Nδ to N7 of the adenine base and accepting one H-bond from the N6 of the exocyclic amino group to its Oδ. **(b)** Q817 recognizes GMP in PDE5A by forming a bidentate H-bond with guanine. The particular orientation of the Q817 side chain is anchored by its H-bond interaction with Q775. The orientation of Q775 side chain is in turn anchored by the H-bond between Nε in Q775 and the carbonyl oxygen in A767 and the H-bond between Oε of Q775 and the Nε of W853. **(c)** Q421 recognizes AMP in the model of AMP bound to PDE1B. **(d)** Q421 recognizes GMP in the model of GMP bound to PDE1B. In (c) and (d), there are no supporting residues to anchor the orientation of the key glutamine residue, this confers PDE1B dual-specificity.

of H-bonds (Figure 3). In PDE4 this glutamine is locked into the cAMP-specific configuration where it forms a bidentate H-bond with the adenine moiety (Figure 3a). This orientation is stabilized by H-bonding to the phenolic hydroxyl group of a nearby tyrosine. In PDE1B there are no supporting residues to anchor the orientation of the key glutamine residue, thus conferring dual specificity for cAMP (Figure 3c) and cGMP (Figure 3d).

Mechanism of cyclic nucleotide hydrolysis

It appears that an invariant aspartate serves as a general base to deprotonate the bridging water molecule, activated by the coordinated Zn^{2+} and Mg^{2+} ions. The resulting hydroxide can act as the nucleophile that attacks phosphorus through an inline associative mechanism (Figure 2d) [6,68,70]. The formation of a pentacoordinate intermediate (or transition state) could be stabilized by the interactions of two phosphoryl oxygen atoms with the metal ions. The O3′ leaving group is protonated by the general acid histidine, found to be H-bonded to the O3′. PDEs commit four amino acids to the donation of one proton, this highlights the importance of the protonation step in the evolution of these enzymes to catalyze the cyclic nucleotide hydrolysis. It has also been suggested that hydrolysis could proceed by a dissociative mechanism [55], leading to the formation of a metaphosphate-like intermediate, where breaking the scissile bond would precede the nucleophilic attack by the hydroxyl group on the phosphorus. This intermediate would be stabilized by the metal ions. The associative and dissociative mechanisms

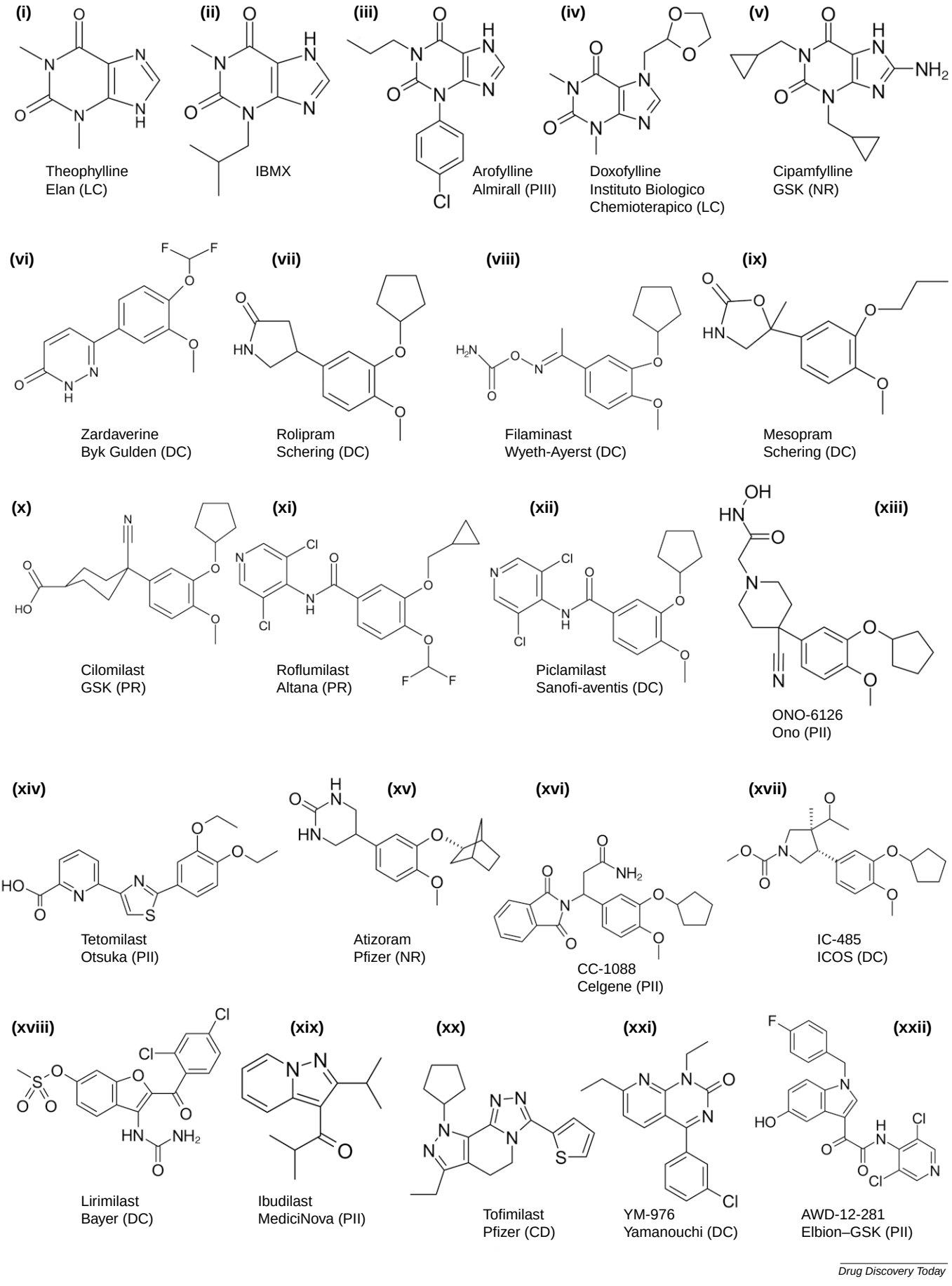
(i)
Theophylline
Elan (LC)
(ii)
IBMX
(iii)
Arofylline
Almirall (PIII)
(iv)
Doxofylline
Instituto Biologico
Chemioterapico (LC)
(v)
Cipamfylline
GSK (NR)
(vi)
Zardaverine
Byk Gulden (DC)
(vii)
Rolipram
Schering (DC)
(viii)
Filaminast
Wyeth-Ayerst (DC)
(ix)
Mesopram
Schering (DC)
(x)
Cilomilast
GSK (PR)
(xi)
Roflumilast
Altana (PR)
(xii)
Piclamilast
Sanofi-aventis (DC)
(xiii)
ONO-6126
Ono (PII)
(xiv)
Tetomilast
Otsuka (PII)
(xv)
Atizoram
Pfizer (NR)
(xvi)
CC-1088
Celgene (PII)
(xvii)
IC-485
ICOS (DC)
(xviii)
Lirimilast
Bayer (DC)
(xix)
Ibudilast
MediciNova (PII)
(xx)
Tofimilast
Pfizer (CD)
(xxi)
YM-976
Yamanouchi (DC)
(xxii)
AWD-12-281
Elbion–GSK (PII)
Drug Discovery Today

FIGURE 4

PDE4 inhibitors and their clinical development status. The chemical structures of a representative set of PDE4 inhibitors are shown with the companies that have developed and/or have conducted clinical trials of these inhibitors as well as their current development status. Abbreviations: LC, launched; PR, pre-registration; PI, Phase I clinical trial; PII, Phase II clinical trial; PIII, Phase III clinical trial; CD, clinical development; DC, discontinued; NR, no development reported.

could represent two extremes but in reality the transition state might lie somewhere in between.

Diversity of PDE4 inhibitor families

Theophylline [Figure 4, structure (**i**)], although only a weak non-selective PDE inhibitor, was the first PDE inhibitor to be used therapeutically and it belongs to a family of xanthine derivatives, which includes 3-isobutyl-1-methylxanthine (IBMX) (**ii**), arofylline (**iii**), doxofylline (**iv**) and cipamfylline (**v**). Although many xanthine derivatives have been developed, and some of them are either under clinical trials [arofylline (**iii**)] or launched [doxofylline (**iv**)], such inhibitors are generally non-selective and relatively weak inhibitors of PDE4.

Rolipram (**vii**) [71] has provided the paradigm for a PDE4 selective inhibitor. Many compounds have subsequently been developed, the dialkoxyphenyl (catechol) family of inhibitors being the largest and the best-characterized. Clinical trials of some of these inhibitors have been conducted for the treatment of asthma but they were discontinued because of narrow therapeutic windows (caused by side effects such as emesis and nausea). These include Rolipram (**vii**), Zardaverine (**vi**), Filaminast (**viii**), Mesopram (**ix**), IC-485 (**xvii**) and Piclamilast (**xii**). Others are currently in clinical trials, such as Atizoram (**xv**), Tetomilast (**xiv**), CC-1088 (**xvi**) and ONO-6126 (**xiii**). Two of the most advanced inhibitors in this catechol class, cilomilast (**x**) and roflumilast (**xi**), have completed Phase III clinical trials and they are currently waiting for regulatory approval as treatments for asthma and COPD [72].

The search for non-emetic PDE4 inhibitors with increased therapeutic windows has led to various, new chemical classes being developed. These include AWD-12–28 (**xxii**), an indole compound currently in Phase II trials for asthma; YM-976 (**xxi**), a pyridopyrimidinone derivative that has been discontinued after Phase I clinical trials; Tofimilast (**xx**), an indazole derivative in clinical development; Ibudilast (**xix**), a pyrazolopyridine compound that has been used extensively in the Asian market both as an asthma controller and as an eyedrop (and is also currently in Phase II clinical trials for multiple schlerosis); and Lirimilast (**xviii**), a benzofuran derivative that has been discontinued following a Phase II clinical trial for asthma.

Structural basis of inhibitor binding to PDE4

Structural analysis of many PDE inhibitor complexes has identified two common features of inhibitors binding to PDEs [54]. A planar ring structure of the inhibitor is held tightly in the active site by a pair of hydrophobic residues forming a hydrophobic clamp and there are H-bond interactions with the invariant glutamine residue, essential for nucleotide selectivity [69]. These two common features define the scaffold of all known PDE inhibitors. However, interactions with residues lining the two hydrophobic subpockets that are close to the invariant purine-selective glutamine are important for inhibitor binding. Furthermore, inhibitor potency can be increased further by exploring interactions with residues near the di-metal ion center as well as through the formation of water-mediated interactions with the metal ions.

There are 30 published co-crystal structures of inhibitors bound to PDE4B or PDE4D and 25 of these are unique complexes. The inhibitors represent four scaffolds: catechol, xanthane, pyrazole and purine analogs. The majority of these co-crystal structures are dialkoxyphenyl derivatives (Figure 5a–c) [52,54,55,70] and only one is a xanthine derivative (Figure 5d) [73]. The superposition of the co-crystal structures of the dialkoxyphenyl family of compounds reveals a scaffold formed by a catechol, H-bonding to the purine-selective glutamine, which is surrounded by the P-clamp (Figure 5). The catechol scaffold superposes extremely well in all of these co-crystal structures, whereas the substituents show significant variations in their binding conformation as well as in the residues that they interact with. The various substituents on the catechol scaffold explore the deep pocket close to the metal binding site and their ability to form interactions with residues lining this pocket determines their relative binding affinity. The relatively smaller pyrrolidinone substituent in rolipram resulted in a relatively lower binding affinity that is similar to that reported for PDE4B as a low-affinity conformer [49]. Both *R*-rolipram and the racemic (*R*–*S*)-rolipram adopt a single binding conformation in PDE4B and PDE4D [54,70]. However, two binding conformations for (*R*–*S*)-rolipram have been observed in one study [55] supporting the notion that PDE4 enzymes can adopt distinct conformations. Notwithstanding this, the true conformation that PDE4 adopts when it binds rolipram with heightened affinity has yet to be unequivocally demonstrated. The more potent dialkoxyphenyl compounds have larger substituents that form more-favorable interactions with residues lining the relatively large M-pocket. The most potent compounds, such as roflumilast and piclamilast, reach deep into the M-pocket, not only interacting with residues near the metal ion but also forming H-bonds with a water molecule that is coordinated to the metal ion. Surprisingly, none of the inhibitors (with the exception of zardaverine) interact directly with the metal ions.

Exploiting crystallography for novel PDE4 inhibitor design

Structural information offers potential in formulating the design of more-potent and more-selective PDE4 inhibitors. Indeed, a scaffold-based drug discovery paradigm has been applied to PDE4 [53]. This begins with the screening of

a low molecular weight compound library to identify low-affinity inhibitors. This is followed by high throughput co-crystallography to select from the compounds that exhibit a dominant binding mode and have appropriate sites for substitution. Such compounds serve as scaffolds for lead optimization. This approach led to the identification of a low-affinity 3,5-dimethyl-1H-pyrazole-4-carboxylic acid ethyl ester (PCEE), revealing the characteristic features of a potential scaffold that binds to PDE4D (Figure 6a). To validate whether PCEE could serve as a scaffold for PDE4, three potential sites of substitution were identified (based on the ability to make favorable chemical interactions in the available space at the active site). A derivative compound, 3,5-dimethyl-1-phenyl-1H-pyrazole-4-carboxylic acid ethyl ester (PhPCEE) was selected for crystallization studies with PDE4D. The studies showed that phenyl substitution at the 1-position of the pyrazole ring does not change the binding mode of the pyrazole in PDE4, thus validating the PCEE moiety as a scaffold for PDE4 (Figure 6b). As a result of analyzing the co-crystal structure >100 compounds were computationally designed and docked into the active site pocket. Substitutions, predicted to cause undesirable interactions with residues in the active site, were eliminated and ten compounds with predicted increased binding affinities were synthesized. A 4000-fold potency increase was obtained [53] in two rounds of chemical synthesis (Figure 6c), validating the usefulness of this approach as a widely applicable strategy to expedite lead discovery efforts.

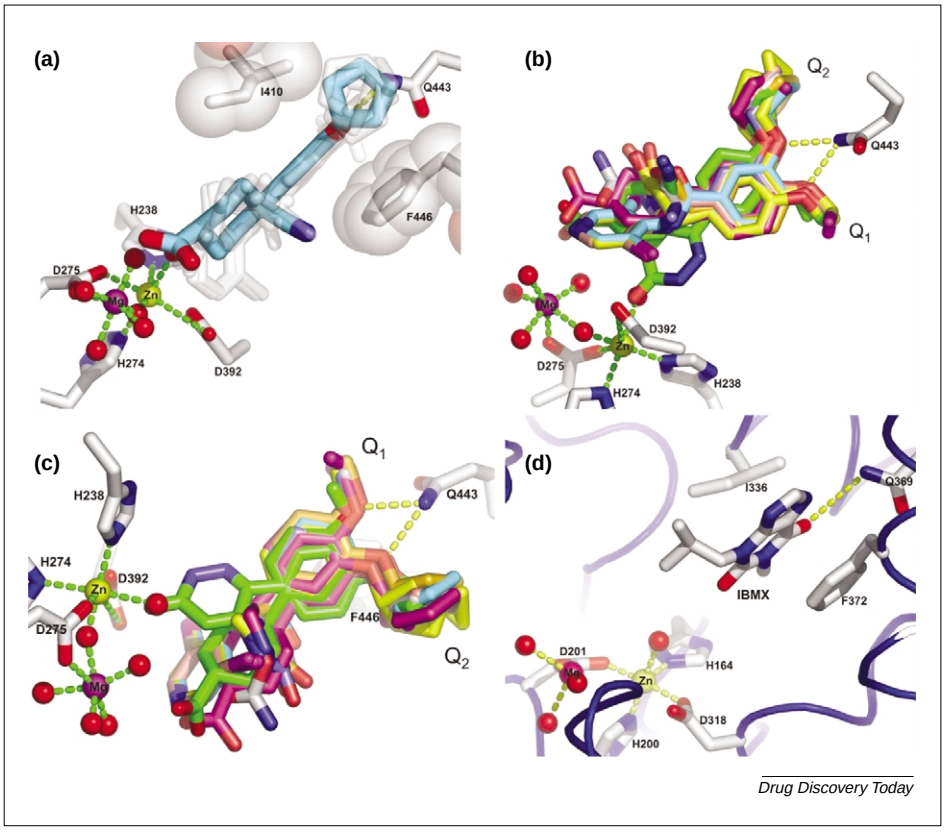

FIGURE 5

Common features of inhibitor-binding to PDE4. (a) Superposition of the structures of all the dialkoxyphenyl compounds bound to PDE4B and PDE4D reveals that the phenyl ring is sandwiched by the hydrophobic clamp formed by two highly conserved residues, I410 and F446 in PDE4B. All the dialkoxyphenyl compounds except cilomilast are represented by semi-transparent stick models shaded in grey. Cilomilast is represented by default atomic colors with the carbon atoms in cyan. Residues I410 and F446 are represented by sticks with semi-transparent sphere models superimposed. **(b)** A view into the phenyl ring 'face-on' which is ~90° rotated along the length of the compounds from (a). The catechol scaffold binding to PDE4 is conserved by forming a hydrogen bond with the purine-selective glutamine Q443 and is sandwiched between the hydrophobic clamp, I410 and F446. **(c)** A view ~180° rotated from that of (b). The catechol scaffold superimposed very well among all the structures. The substituents adopt diverse conformations that exploit the large M-pocket to attain different binding affinities. **(d)** IBMX bound to PDE4D. IBMX is sandwiched by the hydrophobic clamp formed by I336 and F372 and forms a hydrogen bond with Q369. Residues coordinating the metal ions are shown in stick model. The main chain of PDE4D is represented by tubes colored in blue.

PDE4 preclinical pharmacology

Effects of PDE4 inhibition in leukocytes

Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine that is produced largely by monocytes, macrophages and T cells, is an important drug target in rheumatoid arthritis, ankylosing spondylitis, Crohn's disease and psoriasis. TNF-α production by peripheral blood monocytes and T cells is inhibited by rolipram [74,75]. In monocytes this inhibition is accompanied by the elevation of intracellular cAMP and the activation of PKA, effects that are synergistically enhanced by the addition of prostaglandin E_2 (PGE_2), which activates adenylyl cyclase via the prostanoid receptor. Conversely, monocyte production of interleukin-10 (IL-10), an anti-inflammatory cytokine, is enhanced by rolipram in a PKA-dependent manner [76]. These opposing effects on TNF-α and IL-10 production have been explained at the level of the transcription factor (NF-κB), cAMP responsive element binding protein (CREB) and CCAAT/enhancer-binding protein (C/EBP). The PKA activator, dibutyryl cAMP, and the adenylyl cyclase activator, forskolin, inhibit transactivation of the p65 NF-κB subunit (required for TNF-α transcription) [77,78]. By contrast, in monocytes PKA directly phosphorylates and activates CREB and it indirectly activates C/EBP; these actions are important for IL-10 promoter activation [79,80]. Rolipram inhibits IL-12 production in macrophages, although its effects on IL-1β and IL-6 are much less pronounced [81].

Other myeloid cell types (such as neutrophils, eosinophils, basophils and mast cells) participate in the innate immune response by producing cytokines, chemokines and other pro-inflammatory mediators. However, in diseases such as COPD, severe asthma and late-stage rheumatoid arthritis, it is neutrophils that play a key role in the

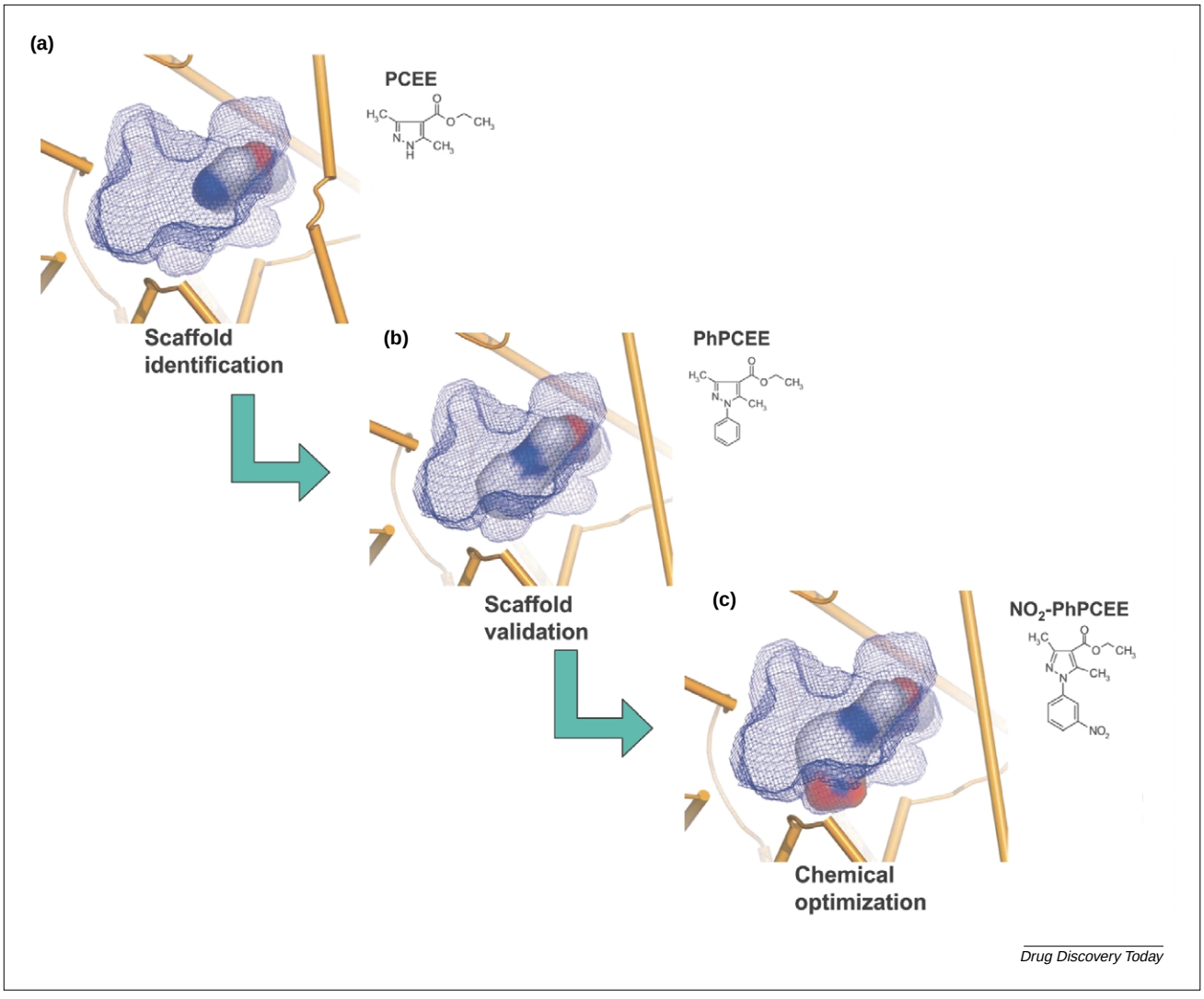

FIGURE 6

Scaffold-based lead discovery of pyrazoles as PDE4 inhibitors. (a) Scaffold identification. The scaffold candidate, 3,5-dimethyl-1H-pyrazole-4-carboxylic acid ethyl ester (PCEE), is a weak PDE4D inhibitor with an IC_{50} of 82 μM (PDE4B IC_{50} = 60μM). **(b)** Scaffold validation. The derivative of the scaffold, 3,5-dimethyl-1-phenyl-1H-pyrazole-4-carboxylic acid ethyl ester (PhPCEE) has significantly increased potency towards PDE4D with an IC_{50} of 0.27 μM (PDE4B IC_{50} = 0.31μM). **(c)** Chemical optimization. The validated scaffold was optimized into a potent PDE4D inhibitor, 3,5-dimethyl-1-(3-nitro-phenyl)-1H-pyrazole-4-carboxylic acid ethyl ester (NO_2-PhPCEE), with an IC_{50} of 0.021 μM (PDE4B IC_{50} = 0.033μM). Compounds are represented by solid surfaces, colored by atomic types. The active site is represented by a blue mesh. The PDE4D is represented by cartoons where helices are shown as cylinders and loops are shown as tubes.

pathological inflammatory process. PDE4 inhibitors suppress multiple neutrophil responses, including the production of IL-8, leukotriene B4 and superoxide anions, degranulation, chemotaxis and adhesion. In allergic diseases such as atopic dermatitis, allergic rhinitis and asthma, eosinophils play a key role. Their superoxide anion production, adhesion and infiltration into airways are inhibited by rolipram. Rolipram inhibits intradermal eosinophil accumulation in models of passive cutaneous anaphylaxis when it is administered systemically, although not when it is administered intradermally, indicating that the targets of drug action are the eosinophils in the circulatory system rather than those in the skin tissue [20,22,72].

T cell signaling through the TCR–CD3 complex appears to be intimately connected to PDE4. Upon ligation the TCRs locate within lipid rafts whereupon Lck is activated, causing the tyrosyl phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) domains within CD3, crucial for full T cell activation [3]. However, TCR ligation effects a transient rise in cAMP, this activates PKA and leads to the phosphorylation and activation of the C-terminal SRC kinase (Csk). Csk activated in this way subsequently phosphorylates and inhibits Lck, attenuating T cell activation. However, stimulation of the co-receptor, CD28, markedly potentiates the recruitment of a β-arrestin–PDE4 complex into T cell lipid rafts upon TCR activation [66]. Delivery of active PDE4 to lipid rafts plays a pivotal role in lowering localized cAMP, causing Csk inactivation and potentiating T cell activation [8,66]. This undoubtedly underpins the profound inhibitory effect

that PDE4-selective inhibitors exert in attenuating T cell function after ligation of the TCR. Additionally, it has been suggested that ligation of the TCR could cause the tyrosyl phosphorylation of PDE4B2 [82], allowing it to associate with CD3ε and thereby reducing localized, inhibitory cAMP levels.

T cell proliferation and production of TNF-α, IL-2, IL-5, IL-4, interferon-gamma and PGE2 are blocked by PDE4 inhibitors [75,83]. This T cell suppressive activity was demonstrated clinically by the PDE4 inhibitor CP80,633 in an atopic dermatitis Phase II trial. CP80,633 administered topically, twice-daily for 28 days caused a significant reduction in erythema, induration and excoriation [84]. This small clinical trial provides the proof of concept that the anti-inflammatory properties of PDE4 inhibition, in this particular case against T cells, can be of significant clinical benefit.

PDE4 inhibitors and mucus secretion in airway disease

Mucus hypersecretion is a key pathological feature that characterizes chronic inflammatory diseases of the airways, such as COPD and asthma. It is proposed that PDE4 inhibition can play a key role in ameliorating this symptom by reducing the transcriptional activity of the predominant gene involved in mucin secretion, *MUC5AC*. It is aberrantly upregulated in asthma and COPD [85]. The preclinical studies that underpin this proposal were performed in the A549 model cell line and it will be interesting to see whether the results translate into human epithelial cells in patients with COPD or asthma.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride ion channel that is activated upon phosphorylation by PKA, which leads to an increase in the probability of channel opening. It is found in pulmonary epithelial cells where it performs a major role in controlling mucociliary clearance [86]. Recently, two independent reports have shown that the functioning of the CFTR in epithelial cells can be selectively regulated by localized PDE4D activity [56,87]. In this regard the CFTR can form a complex with the β_2AR to which AKAP, ezrin and associated PKA are bound [88–90]. This complex bears some analogy with one that was recently identified in HEK293 cells [16], involving the β_2AR, AKAP79 and PKA, to which PDE4D is recruited in complex with β-arrestin upon occupancy of the β_2AR. Such recruited and tethered PDE4D serves to regulate the activity of the AKAP79-bound PKA and its ability to phosphorylate the β_2AR. Such observations exemplify the importance of localization in PDE4 isoform functioning.

Oncology: antitumor and anti-angiogenic effects

The antiproliferative activity of PDE4 inhibitors against T cells and B cells could suggest their potential utility against lymphoid leukemias. In T cell acute lymphoblastic leukemia cells and B cell chronic lymphocytic leukemia cells, rolipram and forskolin induce cell-cycle arrest and apoptosis [91]. Antiproliferative activity by a selective PDE4 inhibitor has also been demonstrated against murine carcinoma cells [92]. In addition to these direct antitumor effects, PDE4 inhibitors block angiogenic responses of endothelial cells (EC). In the human umbilical vein EC proliferation, induced by vascular endothelial growth factor (VEGF), is blocked by the selective PDE4 inhibitor (RP73401) by a process involving suppression of MAPK and cyclin A expression and cell cycle arrest [93]. Rolipram, in combination with salbutamol, inhibits TNF-α-induced E-selectin expression on EC [94] and the PDE4-selective inhibitor Ro 20-1724 has been shown to block VEGF-induced EC migration [95]. These anti-angiogenic effects would not be restricted to the neoplastic setting alone, but also to any non-oncological condition where pathological angiogenesis is driven by VEGF or TNF-α.

PDE4 inhibition has also been shown to ablate Rho-driven migration of fibroblasts on laminin surfaces, which might indicate the potential of PDE4 inhibitors as anti-metastatic agents in certain instances [96].

Diffuse large B cell lymphoma (DLBCL) is a common and often fatal malignancy. It has been noted that an 'expression signature of outcome' in DLBCL is the over-expression of PDE4B in fatal and refractory tumors. This is considered to underpin the resistance of malignant B lymphocytes to cAMP-induced apoptosis, indicating that PDE4 inhibitors could be useful therapeutically [97].

Role of PDE4 and cAMP in the central nervous system

In the CNS PDE4 is expressed in neurons in the cerebral cortex and hippocampus, hypothalamus and striatum, dopaminergic neurons of the substantia nigra and in astrocytes [98–101]. There has been a resurgence in the study of PDE4 in neuroscience [21,102], reminiscent of the period of development of rolipram for depression [103] and Parkinson's disease [104] two decades ago. Indeed, rolipram has been used to demonstrate efficacy in several preclinical models of neurological conditions including depression, memory deficit and spinal-cord injury [21,105,106]. PDE4 is thought to be an integral component in the mechanisms of action for various classes of antidepressants [21]. Chronic treatment of rats with a norepinephrine re-uptake inhibitor, a monoamine oxidase inhibitor, a serotonin re-uptake inhibitor or an atypical antidepressant all resulted in increased PDE4A4 expression [21]. Repeated antidepressant treatment also causes an increase in HARBS expression in the membrane fractions from the hippocampus and cerebral cortex [21].

The selective PDE4 inhibitors, Ro-20,1724 and SDZ-MNS 949, stimulate the uptake of dopamine by rat mesencephalonic neurons *in vitro* and also enhance intracellular dopamine levels in primary rat mesencephalic neurons, in the presence of forskolin [107]. In these studies, elevation of cAMP is caused by the addition of either dibutyryl cAMP or forskolin-protected dopaminergic neurons from the neurotoxic effects of 1-methyl-4-phenyl pyridinium

TABLE 2

Phenotypes of PDE4A, PDE4B and PDE4D knockout mice[a]

Response	PDE4A knockout	PDE4B knockout	PDE4D knockout
Neonatal growth and survival, female fertility	Normal	Normal	Impaired
TNF-α production by circulating leukocytes stimulated by LPS	Normal	90% decrease	Normal
Airway hyperreactivity in response to allergen and cholinergic agonist	Reduced	Reduced	Absent
IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid mediated by allergen	NR	Reduced	Normal
Eosinophil recruitment to bronchoalveolar lavage fluid mediated by allergen	NR	64%	Normal
Neutrophil recruitment to bronchoalveolar lavage fluid mediated by LPS	Normal	31% decrease	48% decrease
Tracheal contractility induced by cholinergic agonist	Normal	Normal	34% decrease in maximal efficacy, fivefold decrease in sensitivity
PDE4 inhibitor-induced shortening of α2 adrenoceptor-mediated anesthesia (behavioral correlate of emesis)	NR	Normal	Reduced
Cognition (long-term memory)	NR	Normal	Increased
Antidepressant behavior	NR	Increased	Increased
Anxiolytic behavior	NR	Decreased	Normal
Myocyte contraction rate mediated by β2 adrenoceptor	Normal	Normal	Increased

[a]References [11–14,36,64,109,112,120]. Abbreviation: NR, not reported.

ion (MPP^+). PDE4 inhibitors reduce dopamine depletion in the striatum and they reduce the loss of tyrosine hydroxylase immunopositive neurons in the substantia nigra of C57BL/6 mice that have been injected with MPTP [108]. Therefore, PDE4 inhibitors have shown efficacy in the MPTP mouse model of Parkinson's disease and, based on *in vitro* studies, the mechanism of action is believed to involve, at least partially, a direct neuroprotective effect.

The processes of learning and memory involve NMDA receptors on hippocampal and cerebral cortical neurons resulting in cAMP elevation via a PDE4-dependent process. Rolipram administration improves working- and reference-memory, impaired by an NMDA receptor antagonist [21,36].

The role of PDE4 subtypes in cellular responses

Differential involvement of PDE4A, PDE4B and PDE4D has been proposed in growth and survival, fertility, airway hypereactivity, inflammatory responses, myocyte contraction and neurological function, based partly on expression patterns in specific tissues and cell types but particularly on the phenotypes of transgenic mouse strains that do not express PDE4A, PDE4B or PDE4D [11–14,109]. Not very much can be inferred about a role for PDE4C simply because it has been studied so poorly, probably because of its more restricted expression pattern.

A summary of animal phenotypes and cellular responses for PDE4A, PDE4B and PDE4D knockout mice is provided in Table 2. From these studies it is notable that circulating leukocytes from PDE4B knockout mice, stimulated *ex vivo* with lipopolysaccharide (LPS), display a 90% reduction in TNF-α production compared with wild-type animals [12]. This might be connected to an observation that PDE4B expression increases in LPS-stimulated monocytes at the transcriptional level [110,111]. By contrast, macrophages from PDE4D and PDE4A knockout mice have been reported to display normal TNF-α responses [112]. In the case of the tracheal smooth muscle contraction, which triggers airway hypereactivity upon stimulation with cholinergic agonists, ablation of PDE4D results in the most profound phenotype. However, in PDE4A and PDE4D knockouts a loss of activity is evident [11]. Neonatal cardiac myocytes from PDE4D, but not PDE4A or PDE4B, knockout mice responded to β-adrenergic receptor stimulation [64].

In general, it appears that PDE4B is uniquely involved in TNF-α and Th2 cytokine production, lung eosinophilia and anxiolytic behavior and PDE4D is uniquely involved in neonatal growth and survival, female fertility, duration of anaesthesia effected by an α_2 adrenoceptor agonist, cholinergic stimulation-induced tracheal smooth muscle contraction, β-adrenergic receptor-induced myocyte contraction and cognition whereas, together, PDE4B and PDE4D are involved in airway hypereactivity, lung neutrophilia and depression. Thus, for inflammatory conditions the profile of a PDE4B-selective inhibitor would appear to be much more desirable. If, however, one were targeting a cognitive deficit the need to inhibit PDE4D would appear to be desirable.

Hardly any information is known, from studies of actual patients, regarding PDE4 subtype expression patterns on cells and tissues. This is clearly an important deficit that needs correcting. However, one study that does address this issue has demonstrated that macrophages from smokers with COPD have elevated levels of PDE4A4 compared with control smokers (without COPD); also PDE4A4 and PDE4B2 levels were higher in the peripheral monocytes of smokers compared with non-smokers [113].

Genetic linkage studies have implicated an association of the PDE4D gene with certain forms of stroke in the Icelandic population but not in various other European populations [114].

The upregulation of PDE4D short isoforms in activated vascular smooth muscle cells suggests a potential for PDE4

inhibitor use in adjunctive pharmacotherapy following percutaneous coronary interventions [115].

Clinical experience with PDE4-selective inhibitors

Rolipram was originally developed as an antidepressant [103] and it was studied in Phase II trials for Parkinson's disease [104]. The efficacy of rolipram was limited by the side effects of nausea and vomiting and it was, therefore, never launched. Since that time the newer generations of PDE4 inhibitors have strived to widen the therapeutic index and it is anticipated that they will eventually gain regulatory approval for COPD, asthma and allergic rhinitis, among others [18,20,22].

A complex variety of factors undoubtedly contribute to the dose-limiting gastrointestinal toxicities of nausea, emesis and diarrhea, which probably include PDE4 subtype selectivity and access to the emetic centers of the brain. However, the anti-inflammatory actions of PDE4 inhibitors make them excellent therapeutic candidates for inflammatory diseases of the pulmonary system, such as COPD [20,22].

Cilomilast [Figure 4 (x)] is the most clinically advanced PDE4 inhibitor, having completed four Phase III COPD clinical trials [72]. The potency of cilomilast is similar to that of rolipram, with a PDE4 IC_{50} of 95 nM and TNF-α production IC_{50} of 110 nM. This compound has also been shown to inhibit allergen-induced IFN-γ and IL-5 production, degranulation of neutrophils and histamine release from human basophils. At the optimal clinical dose of 15 mg b.i.d., cilomilast attains a C_{max}, in healthy volunteers, of 1.3–2.6 μg/ml and has a half-life of 8.2 h. In a COPD dose-ranging safety and efficacy study, cilomilast dosed at 15 mg b.i.d. significantly improved spirometry measurements. Nausea (11% versus 1% for placebo), diarrhea (9% at 15 mg doses versus 1% for placebo) and abdominal pain (8% versus 3% for placebo) were the most commonly reported adverse events. In four pivotal 24-week COPD trials, cilomilast demonstrated a consistent improvement for FEV_1 of ~30 ml, achieving statistical significance in two out of the four studies. GlaxoSmithKline received an approved letter from the FDA for cilomilast in September 2003 but its status since that time has not been publicly disclosed.

Roflumilast [Figure 4 (xi)] followed cilomilast into the clinic and it is in development for asthma as well as COPD [72]. It is ~100-fold more-potent than cilomilast at the enzymatic level, with a PDE4 IC_{50} of 800 pM. It inhibits neutrophil, eosinophil, monocyte, macrophage, dendritic cell and T cell responses with IC_{50}s in the 1–20 nM range. It also inhibits TNF-α production in human whole blood with an IC_{50} of 50 nM. It is active in models of allergen-induced bronchoconstriction and lung inflammation *in vivo*, as measured by inhibition of eosinophilia and TNF-α production. It has also been shown to inhibit subepithelial collagenization and thickening of the airway epithelium. The oral bioavailability of roflumilast in humans is 79% and, *in vivo*, 90% is converted to an N-oxide metabolite with similar *in vitro* potencies as the parent drug. The relatively long half-lives of roflumilast and its N-oxide metabolite (10.3 h and 19.6 h, respectively) enable once-a-day dosing. In a clinical trial of allergic rhinitis 500 μg o.d. of roflumilast improved rhinal airflow, itching and rhinorrhea. The same dose was also efficacious in an exercise-induced asthma study. In a 12-week asthma trial versus montelukast (a leukotriene D4 receptor antagonist), roflumilast (500 μg o.d.) increased the mean FEV_1 by ~300 ml, a significant improvement over montelukast. In a 12-week asthma trial compared with 400 μg b.i.d. beclomethasone dipropionate (BDP, corticosteroid), 500 μg o.d. roflumilast improved FEV_1 and FVC by ~300 ml and the use of rescue medication and symptom scores were significantly reduced (effects similar to that of BDP). In an allergen challenge asthmatic airway response trial, 250 and 500 μg of roflumilast dosed for 7 or 10 days attenuated the late asthmatic reaction drop in FEV_1 by 27% and 43%, respectively, whereas the early asthmatic response was attenuated by 25% and 28%, respectively [116]. COPD trials with roflumilast include a 12-week withdrawal trial and a 24-week trial in 1413 patients (RECORD study). In the RECORD study at the 500 μg dose, roflumilast increased FEV_1 by 97 ml after 24 weeks compared with the placebo. Quality of life, as measured by a drop in the St George's Respiratory Questionnaire score of 3.51 points (a change of four is considered clinically relevant), was improved. The most common adverse events in the COPD study at the 500 μg dose included diarrhea (6.1%), nausea (3.2%), headache (1.8%) and abdominal pain (1.6%). There were no reports of vomiting. Altana filed an application for the use of roflumilast for asthma and COPD in Europe in February 2004 and they are expected to file a similar application in the USA. Because of slower than expected enrolment in the extensive Phase III clinical trials, the application for US approval will be filed after the first half of 2005 (Altana Press Release 28 October 2004).

IC485 [Figure 4 (xvii)], an orally administered PDE4 inhibitor, had been in a Phase II COPD trial that began in December 2003 but, because of insufficient efficacy, its development has been stopped (ICOS press release). In a Phase I trial in healthy volunteers ONO-6126 [Figure 4 (xiii)], administered at doses of 3–9 mg b.i.d for seven days, inhibited *ex vivo* TNF-α production by peripheral blood in a dose-dependent manner [117]. In 2004 ONO-6126 entered a placebo-controlled three month Phase II COPD trial. CC-10004 (Celgene), an oral PDE4 inhibitor (PDE4 IC_{50} = 74 nM, TNF-α IC_{50} = 110 nM) demonstrated tolerability in a Phase I multiple dose safety and pharmacokinetic study (up to 20 mg o.d.) with no increase in adverse events and plasma levels and it reached a C_{max} approximately ninefold higher than its whole blood TNF-α IC_{50}. CC-10004 is currently in Phase II trials for asthma and psoriasis. AWD 12–281 [Figure 4 (xxii)] [19] is unique because it was developed for administration by inhalation. In Phase I studies, administered as a dry powder, it demonstrated tolerability

up to 40 mg per day with adverse events indistinguishable from the placebo. AWD 12–281 has been in Phase II studies of allergic rhinitis, bronchial asthma and COPD.

Two novel PDE4 inhibitors in Phase I development for neurological disorders are MEM-1414 and ND1251. MEM-1414 (Memory Pharmaceuticals, www.memorypharma.com/pipeline.html) is being targeted toward Alzheimer's disease, mild cognitive impairment and depression. ND1251 (Neuro3D) has shown significant antidepressant activity in the mouse and rat and it is being clinically developed for depression, with Phase I clinical trials underway.

Conclusions

The plethora of PDE4 isoforms and their widespread expression pattern could be looked upon as a strength and a weakness of PDE4 as a target for drug development. A major challenge will be to refine PDE4 inhibitors to maximize therapeutic efficacy in specific disease states. Thus, future developments are probably going to focus on the ability to target specific PDE4 subfamilies and isoforms. By doing these structural analyses, PDEs are positioned to play a key role in the identification of subfamily-specific inhibitors.

The availability of crystal structures of the catalytic domain of several PDE4 isoforms has offered a great opportunity for the rationale design of isoform-specific PDE4 inhibitors. However, the high degree of structural similarity between the active sites of various PDE4 isoforms also poses a tremendous challenge. The differences in the regulatory domains of various PDE4 isoforms might be exploited for the design of isoform-specific PDE4 inhibitors and this effort will be greatly facilitated by the crystal structure of full length PDE4 isoforms. Additionally, novel approaches are required to target specific PDE4 isoforms. Regarding this, it is now appreciated that the location of particular signaling complexes at distinct sites within cells, leading to functional compartmentalization, is key to the action of all signaling pathways. This is exemplified in PDE4 where dominant–negative strategies have provided a proof of principal for 'displacement' of specific PDE4 isoforms that have defined functional outputs. Thus, in the case of PDE4, disrupting targeting provides a potential new route for drug discovery where isoforms have identical catalytic sites and are, therefore, similarly sensitive to active-site-directed inhibitors. The difficulty of disrupting pre-existing protein–protein interactions will depend upon the affinity of association. When this is high, disruption of pre-formed complexes will prove to be very difficult. However, molecules aimed at masking interaction sites on newly synthesized targets will provide a useful strategy, especially in diseases where chronic therapeutic treatment is the standard. Such a route, together with siRNA-mediated ablation, could provide a way of generating PDE4 isoform therapeutics for future highly targeted actions that prevent the inhibition of all the members of PDE4 families.

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