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Thiophene inhibitors of PDE4: Crystal structures show a second binding mode at the catalytic domain of PDE4D2

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ABSTRACT

PDE4 inhibitors have been identified as therapeutic targets for a variety of conditions, particularly inflammatory diseases. We have serendipitously identified a novel class of phosphodiesterase 4 (PDE4) inhibitor during a study to discover antagonists of the parathyroid hormone receptor. X-ray crystallographic studies of PDE4D2 complexed to four potent inhibitors reveal the atomic details of how they inhibit the enzyme and a notable contrast to another recently reported thiophene-based inhibitor.

Cyclic 3′,5′-Nucleotide phosphodiesterase family 4 (PDE4) inhibitors have been identified as therapeutic targets in a variety of conditions, particularly inflammatory diseases.[1] While numerous compounds have not advanced past the clinic, Roflumilast (Daxas®, Nycomed), was recently launched in Europe and is due for release (as Daliresp™, Forest Laboratories) in North America in late 2011 for the treatment of chronic obstructive pulmonary disease (COPD).[2] The side effect profiles of many PDE4 inhibitors related to the archetypal compound rolipram have triggered interest in the development of novel chemotypes that may exhibit an improved therapeutic window.[1,3] In addition, a number of structures have been obtained of PDE4-ligand co-crystals, providing the opportunity for structure-based optimization, with respect to sub-type selectivity, ADME, toxicity and other important pharmaceutical properties.[4,5]

We report on thiophene-based PDE4 inhibitors, identified serendipitously while searching for antagonists of parathyroid hormone-related protein (PTHrP) interaction with its receptor. After determining the crystal structure of PTHrP bound to a neutralizing antibody, we reasoned that the antibody might be mimicking how the PTH1 receptor might interact with the hormone.[6,7] Thus we used the structure to search a virtual library of 2 million compounds for small molecule antagonists that might be developed into drugs to treat humoral hypercalcemia of malignancy and bone metastasis formation. The assays utilized a cre-luciferase reporter assay in a PTH/PTHrP-responsive osteosarcoma cell line, UMR106, which effectively measures the increase in cAMP in response to the GPCR agonist. In this assay, the effect of ligands in regulating the cre-luciferase response of cells to treatment with PTHrP was monitored. A large number of compounds yielded marked activation of the luciferase activity and amplified the response of PTHrP itself. One identified possible mechanism for this increase was inhibition of cellular PDE activity that would suppress cAMP breakdown, so these compounds were then assessed for PDE inhibition using lysates from the same cell line (Fig. 1a). While around 15 compounds showed some level of PDE inhibition, a series of six thiophene derivatives (**1**–**6**, Fig. 1b) from the compound library were identified as PDE inhibitors, and showed comparable inhibition to rolipram (IC_{50} = 0.6 μM), implicating PDE4 as the responsible isoform.[8] The compounds also inhibited PDE4 isolated from U937 cells (data not shown). Compound **5** was the most potent and found to have an IC_{50} of approximately 100 nM in these cell lines (Table 1).

We sought to determine the elements contributing to inhibitory potency towards PDE4 by preparing a range of analogues. Our general synthetic route, shown in Scheme 1, gave us the

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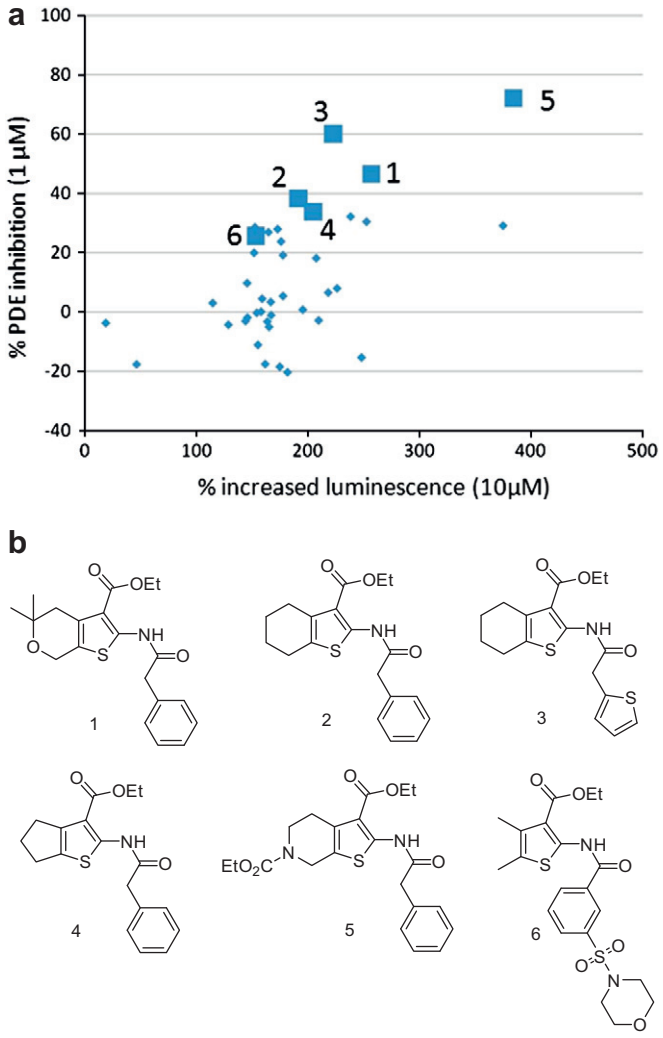


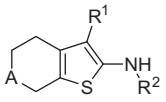
Figure 1. (a) Plot of activities for screening compounds in cre-luc reporter assays and PDE inhibition assay in UMR106 cell lines. Methods described in Supplementary data. (b) Chemical structures of thiophene based PDE4 inhibitors **1–6** identified by screen.

opportunity to examine the role of the fused ring, the arylacetamide and the ester functions of the thiophene compounds. Compounds **2**, **5**[9] and analogues **10a–t** could be prepared by varying the acylating reagent, the ketone or the malonitrile ester derivative used (Scheme 1, Table 1, see also Supplementary data).

Where *N*-Boc-piperid-4-one was the starting ketone, the Boc-protected product could be readily deprotected with trifluoroacetic acid to yield the secondary amines **11** and then further derivatized either as carbamate, acetyl or urea derivatives **12a–d** (Scheme 2, Table 1). For a full description of the compounds prepared, see the Supplementary data.

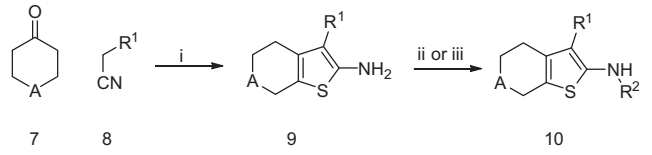
The compounds were screened in isolated enzyme assays using the scintillation proximity assay (SPA) format with recombinant human (rh)PDE4D2 overexpressed from E. coli or against PDE isolated from UMR106 cell lysates. We commenced our studies by examining the activity of our inhibitors in the UMR106 osteosarcoma cell line. These cells have been shown to be predominantly PDE4 isoforms.[8] When compared to the results using the recombinant PDE4D2, we did see a change in the rank order of potency of some compounds. This is not unexpected as inhibitory potency may be a function of isoform, splice variant and in the case of recombinant forms, the presence of regulatory domains.

Table 1
PDE4 inhibition by thiophene derivatives

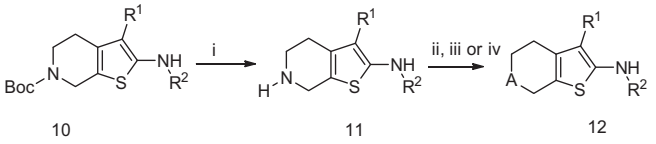


Number	A	R^1	R^2	Inhibition at 1 µM[a] (%)	IC_{50}[a] (µM)
2	CH_2	CO_2Et	$PhCH_2CO$	68	1.5
5	N–CO_2Et	CO_2Et	$PhCH_2CO$	86 (85)	0.10 (0.10)
10a	CH_2	$CONH_2$	$PhCH_2CO$	(15)	
10b	CH_2	CN	$PhCH_2CO$	(24)	
10c	N–CO_2Et	CO_2Et	2-Pyridinylacetyl	72 (73)	(0.08)
10d	N–CO_2Et	CO_2Et	2-Thiophenylacetyl	98 (70)	0.022 (0.182)
10e	N–CO_2Et	$CONH_2$	$PhCH_2CO$	−1 (18)	
10f	N–CO_2Et	CO_2Et	PhCO	(14)	
10g	N–CO_2Et	CO_2Et	$PhCH_2$	(33)	
10h	N–CO_2Et	CO_2Et	4-Ms-$PhCH_2CO$	(−4)	
10i	N–CO_2Et	CO_2Et	2-MeO-$PhCH_2CO$	(37)	
10j	N–CO_2Et	CO_2Et	3-MeO-$PhCH_2CO$	(9)	
10k	N–CO_2Et	CO_2Et	4-MeO-$PhCH_2CO$	23 (26)	
10l	N–CO_2Et	CO_2Et	$PhCH_2CH_2CO$	(22)	
10m	N–CO_2Et	CO_2Et	3-Pyridinylacetyl	−20	
10n	N–CO_2Et	CO_2-c-hexyl	$PhCH_2CO$	61	
10o	N–CO_2Et	CO_2-c-pentyl	2-Thiophenylacetyl	95	0.290
10p	N–$CO_2{}^tBu$	CO_2Et	$PhCH_2CO$	38	
11a	N–H	CO_2Et	$PhCH_2CO$	(25)	
12a	N–Ac	CO_2Et	$PhCH_2CO$	(50)	(1.0)
12b	N–C(O)$NHCH_2Ph$	CO_2-c-pentyl	2-Pyridinylacetyl	65	
12c	N–C(O)NHEt	CO_2-c-pentyl	2-Thiophenylacetyl	88	
12d	N–C(O)NHEt	CO_2Et	$PhCH_2CO$	98	0.027

[a] Values from assay of rhPDE4D2. Values from UMR106 lysates are given in parentheses (see Supplementary data for methods).



Scheme 1. Reagents and conditions: (i) S8, morpholine; (ii) R2-X; (iii) RCHO, NaCNBH3.



Scheme 2. Reagents and conditions: (i) TFA/DCM, rt, 1 h; (ii) Ac2O, (iii) Cl-CO2R3; (iv) R3-NCO.

The PDE4 assays (Table 1) identified the three key elements for potent inhibition: an ester at the 3-position (R^1), an aryl- or heteroarylacetamide (not substituted) at the 2-position (R^2) and a piperidinyl carbamate or urea on the fused ring (A). In general, compounds with fused piperidine rings (such as **5**) were preferred over the carbocycles (such as **2**). Esters substituents (methyl to cyclohexyl) were active, while amides and nitriles were not. The phenylacetyl group could not be substituted, lengthened or shortened without diminished activity, but compounds with heteroaryl

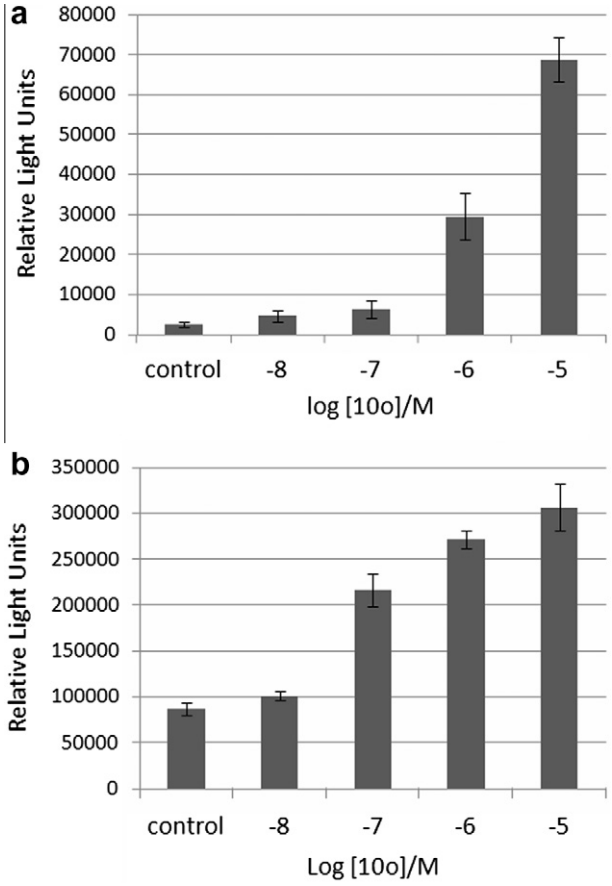


Figure 2. Dose dependent activation of luciferase activity by **10o** in transfected UMR106 cells. (a) Luminescence (relative light units) due to treatment of cells with **10o** alone at 10–10000 nM. (b) Treatment of cells with 1 nM PTHrP plus **10o** at 10–10000 nM. Methods described in Supplementary data.

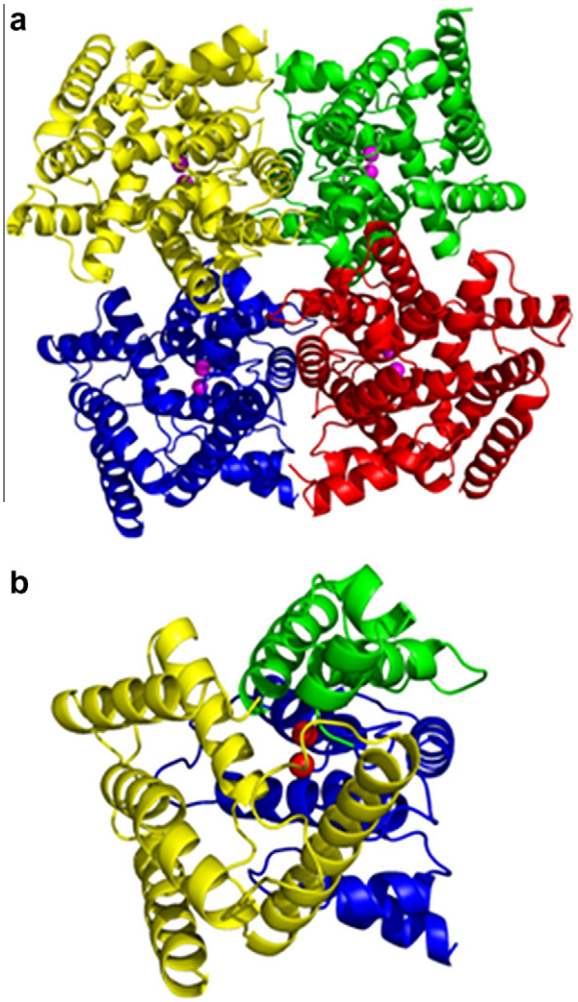


Figure 3. Crystal structure of the catalytic domain of PDE4D2. (a) The tetramer. Monomers A, B, C and D are colored in yellow, green, red and blue, respectively. Zn ions are shown as magenta spheres. (b) The monomer. Helices 1–7 are colored in blue, helices 8–11 are colored in green and helices 12–16 are in colored yellow. Zn ions are in red.

groups, such as 2-thiophenacetyl (**10d**) and 2-pyridinylacetyl (**10c**) showed strong PDE4 inhibition. Finally, the carbamate could be substituted by ureas, (**12c, 12d**) but other changes were unfavorable (**12a**).

The cellular activity of the compounds was demonstrated by the use of the reporter assay described above. For example, **10o** showed potent dose-dependent amplification of the luminescent readout both from basal and PTHrP-stimulated cells. (Fig. 2) The other compounds of the series also showed cellular effects consistent with the observed PDE4 inhibition. These data yielded a picture of a molecular interaction of ligands with the enzyme that was well optimized. A binding model was proposed analogous to ethyl ester derivatives described by Card et al.[10] where the invariant, purine-scanning glutamine of PDE4 interacted with the ethyl ester group, and the thiophene was acting as an isostere for the purine ring of cAMP. Beyond that the binding pose at the active site was not obvious.

The catalytic domain of PDE4D2 was expressed, purified and crystallized using modifications of the methods published by Huai et al.[11] The crystal structure of *apo*-PDE4D2, determined at 2.1 Å resolution, is composed of four monomers assembled into a tetramer in the asymmetric unit (Fig. 3a). The monomer structures were largely identical to those previously described with 16 α-helices divided into three sub-domains.[11] The first sub-domain is composed of α-helices H1–H7, the second is formed by α-helices H8–H11 and the third sub-domain consists of α-helices H12–H16 (Fig. 3b). The intersection of the three sub-domains forms the binding site for substrates and inhibitors.[4,11,12] Two Zn ions were found within the active site. We were also successful in crystallizing the enzyme in complex with four thiophene inhibitors; **10d**, **10o**, **12c**, **12d**. The overall structures of the complexes were very similar to the structure of apo-PDE4D2, with no overall significant conformational changes or any significant perturbations of residues in the binding pocket due to the binding of the ligands. Superposition of the tetramers of the **10d**, **10o**, **12c**, **12d** complex structures onto the apo structure, gives a rmsd between the alpha carbons of 0.6, 0.4, 0.3, and 0.3 Å, respectively. There is some variation in the orientation of the side-chain of the active site residue Met 357 in all structures. This amino acid appears to be flexible and does not have electron density present in all monomers. The PDE4 inhibitors were located in the nucleotide binding pocket and have fundamentally identical binding poses (Fig. 4). Overall there are between 60 and 72 van der Waals contacts between the protein and the ligands. The best inhibitors of the PDE4D2, **12d** and **10d**, show the most clearly defined electron density.

As anticipated, the carbonyl of the ester group for each of the inhibitors forms the key interaction with Gln 369, and the ethyl or cyclopentyl groups are located in a small hydrophobic pocket lined by Met 337 and Met 357. The ester carbonyl, acetamide and thiophene form a planar surface for a stacking interaction with Phe 372 (Fig. 4a–d). The orientation of the aryl ring varies slightly in the four structures to accommodate differences in ligand

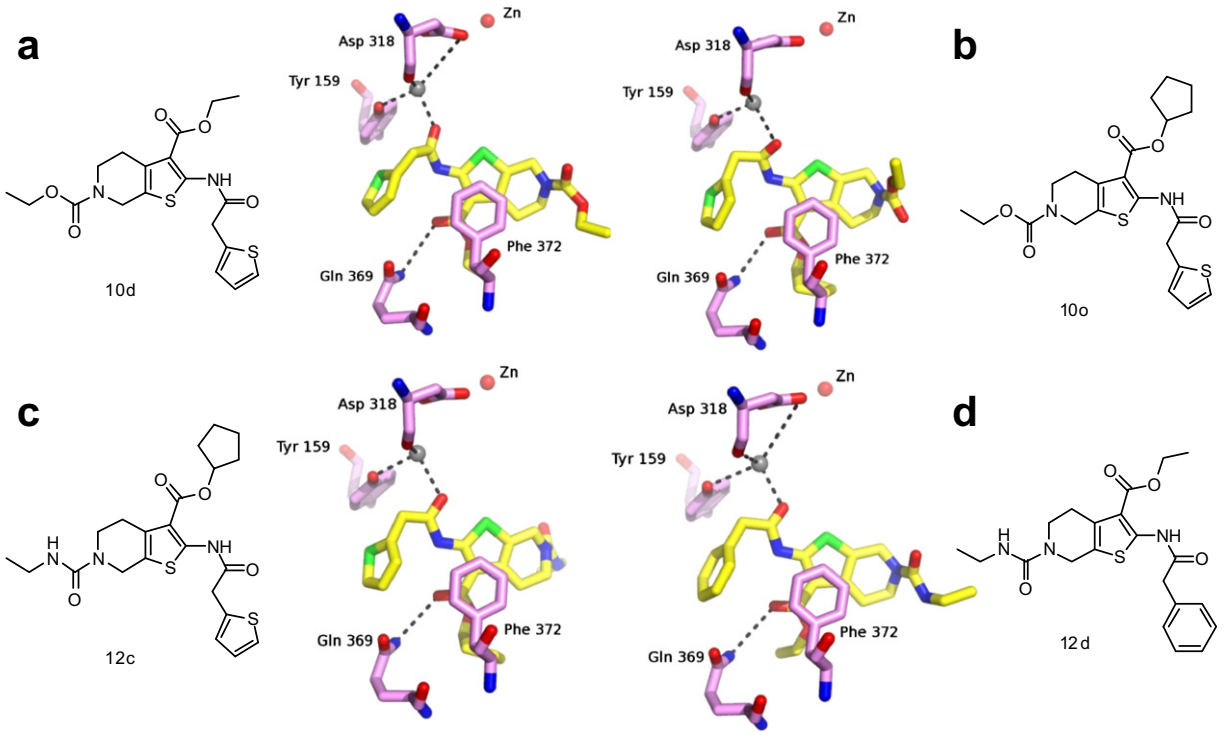


Figure 4. Structures and binding mode of each compound in the active site of PDE4D2. (a) Compound **10d**, (b) Compound **10o**, (c) Compound **12c** and (d) Compound **12d**. Water molecules are shown in grey. PDB codes: Apo form: 3SL3; **10d**: 3SL4; **10o**: 3SL8; **12c**: 3SL6; **12d**: 3SL5.

binding. The carbonyl of the 2′-acetamide of the thiophene forms water-mediated hydrogen bonds to the side-chain of Tyr 159 in all cases and water-mediated hydrogen bonds to the main- and/or side-chain oxygens of Asp 318, depending on the ligand and monomer subunit. The arylacetamide group is positioned deep in the binding pocket, with the 2′-pendant aryl ring folding back towards the ester group, filling a space in the nucleotide binding pocket not previously occupied by any reported ligand in a PDE4 crystal structure.

The influence of the carbamate and the urea substituents, which are important to inhibitory potency, is not obvious from the four crystal structures. Indeed, the density for the ethyl group is poorly defined in the carbamate of both **10d** and **10o** and the urea derivatives, **12c** and **12d**. Depending on the ligand and monomer unit, the carbonyl can project either towards Ile 336 (**10d**), or Ile 376 (**12c**, **12d**), whereas in other (**12c**) monomer subunits it is orientated towards residues Phe 340 and Cys 358.

The structure–activity relationships were in excellent agreement with the crystal structures in several ways. Firstly, the importance of the ester function to activity is apparent as it makes a pivotal hydrogen bond with the carboxamide of the conserved Gln 369, which has been a hallmark of numerous inhibitors such as rolipram and roflumilast and also the pyrazole esters described by Card et al.[10] Interestingly, the ortho placement of the ester and the acetamide on the thiophene ring makes a planar arrangement that stacks against the ring of the phenylalanine. The ethyl or cyclopentyl groups of the ester fill the space occupied by the cyclopentyl ring of rolipram. The carbonyl of the acetamide makes hydrogen bond networks via water molecules to an aspartyl residue, Asp 318.

Second, the specific requirement of the arylacetamide is explained by its folded conformation in the binding pocket, which would not be expected to tolerate shortening to an arylamide or lengthening to a phenethylcarboxamide chain. The poor activity of substituted-phenyl derivatives is also explained in this context as there is very little available space. The location of the

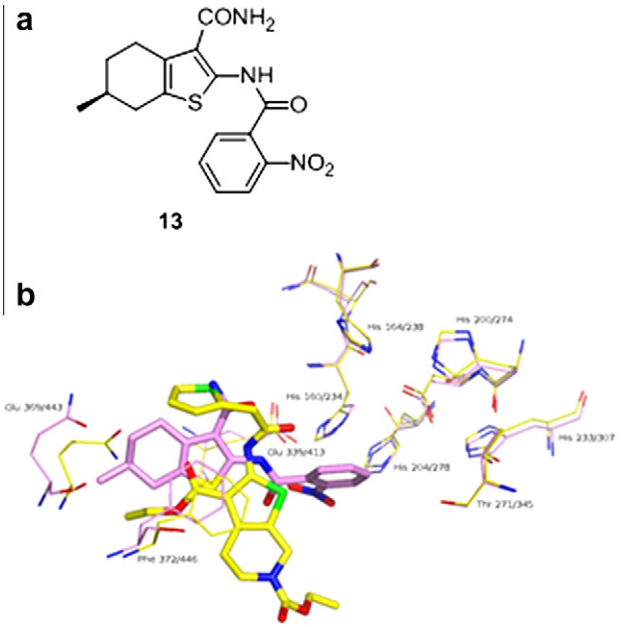


Figure 5. Thiophene complex crystal structures. (a) Structure of reported tetrahydrobenzothiophene inhibitor, **13**[14] (b) superposition of PDE4B/D thiophene complex crystal structures: yellow **10d**, magenta: **13** PDB:3HMV.

arylacetamide in the binding pocket is also of interest. Comparison to all reported PDE4 structures described to date shows that this is an unprecedented binding motif for PDE4 inhibitors.

What is less well explained by the crystal structures is the improved potency of the ethyl carbamate and ethyl urea derivatives relative to the carbocycle in **2**. The generally poor density and variable positioning of these groups suggests flexibility at this region. The improved potency may best be explained as a presentation of a

more hydrophilic region to the aqueous environment at the binding pocket entrance.

Although the thiophene class and particularly the 2-amino-3-carboxythiophenes have been shown to display a number of biological activities,[13] our identification of PDE4 inhibition by this thiophene class coincided with workers at Glaxo who also published a series of analogous thiophene derivatives, but interestingly the compounds showed markedly different SAR to our own series and in particular were poor inhibitors of PDE4D relative to PDE4B.[14] Consistent with that, the crystal structure of PDE4B complexed to the GSK inhibitor **13** shows that the thiophene molecule binds in a completely different orientation, (Fig. 5), with virtually no overlap of the compounds in the active site. So while the structural analogy of the inhibitor is obvious, they share nothing in common from the view of the PDE4 catalytic site.

In conclusion, here we have shown the structure–activity relationships (SAR) and crystallographically determined binding poses for a series of novel PDE4 inhibitors, one of which was discovered serendipitously via a cre-luciferase based reporter assay. The class falls into the category of non-rolipram related PDE4 inhibitors, which may be of some significance given the perceived association between the rolipram class and a narrow therapeutic window. The compounds show an interesting and in some ways unprecedented binding interaction as observed crystallographically, that is supported by SAR studies. We hope to use this information further in the development of clinically or pharmacologically useful PDE4 inhibitors.

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Supplementary data

Supplementary data (selected enzyme inhibition curves, crystallographic data and experimental procedures) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.109.

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