Chapter 5


Manuscript in preparation
Abstract

The *Trypanosoma brucei* phosphodiesterase B1 (TbrPDEB1) enzyme has been validated as a target for the treatment of Human African Trypanosomiasis (HAT). The most promising published TbrPDEB1 inhibitors continue to show a selectivity for human PDE4. Compounds are needed that form interactions specific to TbrPDEB1 in order to achieve selectivity for TbrPDEB1. With the help of X-ray crystallography we are able to show that it is possible to achieve selectivity by addressing the parasite specific P-pocket in TbrPDEB1. The first liganded TbrPDEB1 crystal structures presented here provide insights into the lack of selectivity observed in TbrPDEB1 inhibitors developed so far. Using structural information as a basis, scaffold hopping enabled the development of the first potent selective TbrPDEB1 inhibitors.
5.1 Targeting HAT through PDE inhibition

Human African Trypanosomiasis (HAT) is a Neglected Tropical Disease (NTD) endemic to sub-Saharan Africa where an estimated 70 million people are at risk of infection.\textsuperscript{1} Transmitted by infected tsetse flies, *Trypanosoma brucei* parasites enter the blood, proliferate, spread, and eventually reach the brain. Such an infection is usually fatal without treatment.\textsuperscript{2} Patients are at risk of severe side effects and sometimes even death following treatment, with antiquated drugs still being used as second-line treatments or treatments for infection with the less common variant *T.b.rhodesiense*.\textsuperscript{3} A large animal reservoir maintains the threat of HAT even as patient numbers fall to their lowest numbers in decades.\textsuperscript{4} New treatments are required to sustain recent successes in controlling the disease and to reduce the burden of treatment on patients.

Phosphodiesterases (PDEs) have been validated as drug targets for the control of trypanosomes.\textsuperscript{5} Proliferation of the parasites is halted through simultaneous inhibition of the activity of both TbrPDEB1 and TbrPDEB2.\textsuperscript{6} The TbrPDEB enzymes play a role in cytokinesis and inhibition leads to a phenotype with multiple nuclei and kinetoplasts and the inhibition of parasite proliferation.\textsuperscript{7} Although both TbrPDEB1 and TbrPDEB2 must be inhibited to control parasite levels, a sequence identity of 77\% across the full sequence and 88\% across the catalytic domain and the similarity in the inhibitory activity of most inhibitors allows drug discovery efforts to initially focus on just one of the enzymes when identifying novel inhibitors.

The TbrPDEB enzymes can be inhibited competitively with ligands binding to the substrate binding pocket, thereby blocking the hydrolysis of cAMP to AMP.\textsuperscript{6} Key elements of the TbrPDEB binding pockets that are required for catalytic activity are conserved across all PDEs; a metal binding region that contains two metal ions, a hydrophobic pocket to bind the aromatic adenosine ring system and a glutamine residue that forms multiple hydrogen bonds with the cyclic nucleotide substrates.\textsuperscript{8} This leads to a highly conserved fold across all PDE subtypes irrespective of the substrate specificity of a PDE for cAMP or cGMP (see Chapter 2).
The structural similarity of PDEs has consequences for the selectivity of inhibitors, many of which are promiscuous, inhibiting several different PDE subtypes.\(^9\) This overlapping activity of PDE inhibitors is a boon for the discovery of inhibitors of PDE classes that are less well studied. The first TbrPDEB1 inhibitors were discovered by screening known human PDE inhibitors.\(^6,7,10\) Especially PDE4 inhibitors were found to display significant activities against TbrPDEB1. In the SAR studies carried out around the PDE4 inhibitor 1 (piclamilast), and the PDE5 inhibitors sildenafil and tadalafil, 1 remained the most potent TbrPDEB1 inhibitor.\(^10-12\) Overall, the most potent inhibitor of TBrPDEB1 known to date, compound 2, was initially synthesized during an optimization of PDE4 inhibitors and later discovered to inhibit TbrPDEB1 during an HTS of in-house compounds at Nycomed.\(^5\) The related compounds 3 and 4 were discovered through a combination of FBDD and scaffold hopping (Chapter 4) and were developed with PDE4 inhibitors as starting points.\(^7\) An undesired consequence of developing TbrPDEB1 inhibitors from human PDE inhibitors is the inherent activities of the scaffolds on human PDEs (mainly human PDE4).

![Figure 1: Structures of PDE inhibitors and their activities against TbrPDEB1 and PDE4 (1\(^{10,13}\), 2\(^5\), 3 - 4\(^{14}\), 5\(^{10,15}\), 6\(^{11,16}\) and 7\(^{17}\)).](image)

Significant side effects pose a challenge during the drug development of PDE4 inhibitors, with nausea and emesis proving the most difficult to avoid.\(^18\) After two decades of PDE4 research, compound 5 (Figure 1, roflumilast) was the first PDE4 inhibitor to be approved as a drug.\(^19\) This presents a particular problem for TbrPDEB inhibitors showing greater activity on PDE4, since HAT patients may already be weak and high dosing may be required to clear the trypanosomes. This most likely rules out the possibility of using compounds optimized for PDE4 to treat HAT. Instead compounds selective for TbrPDEB above human PDEs need to be identified for development. Structure based virtual screening has provided...
the first compounds with improved selectivity indexes for TbrPDEB1 over PDE4 (Chapter 4). However, with micromolar potency and near equipotency, these compounds lack important attributes needed from TbrPDEB1 inhibitors to be used as drugs to treat HAT.8 The discovery of the first series of compounds, which overcome these challenges is presented in this chapter.

5.2 Targeting the P-pocket

A subpocket dubbed the P-pocket is found in crystal structures of TbrPDEB1 (Chapter 4), LmjPDEB1 and TcrPDEC, but not in crystal structures of human PDEs and may provide a handle for selectivity.8,20,21 It is located adjacent to the conserved glutamine residue Q874 and extends from the Q2 pocket through a gap between helices H14 and H15, forming a pore through the protein to the water layer on the opposite side (Figure 2). Several key residue differences account for the appearance of the P-pocket in TbrPDEB1 and not in PDE4. In TbrPDEB1 T841 occupies a smaller volume, where M411 in PDE4 extends across the back of the Q2 pocket. The π-π interaction between F844 and F862 (C423 in PDE4) holds the conformation of the M-loop and the additional M-Loop residue (M868) not found in PDE4 extends and repositions the M-Loop, providing space for the P-pocket. Compared to TbrPDEB1 the P-pocket in LmjPDEB1 overlaps closely, while in TbrPDEC the P-pocket is displaced and smaller due to a shorter M-Loop (2 residues) and a leucine at the position of T841.
Figure 2: A) The P-pocket of TbrPDEB1 (4I158) shown as a gap extending from the Q2 pocket in the gray surface. B) The same region in PDE4 (1XM422) showing there is no gap in the surface, however the indentation of the Q2 pocket is visible.

Compounds have been proposed that might address the P-pocket with a flexible group extending from the core scaffold, for example 2, 3, 4, 68 and 717. Compounds 3 and 4 for example can be docked in TbrPDEB1 with conformations that allow the tetrazole tail of the compounds to extend into the P-pocket (Chapters 3 and 4). However, all of these compounds fail to show selectivity against PDE4 in published data (Figure 1). To resolve this issue and to obtain molecular insights in the binding of the reported TbrPDEB1 inhibitors we decided to incorporate structural biology studies in our drug discovery pipeline. Optimizing the x-ray crystallography protocols developed in Chapter 4, we were able to produce ligand bound crystal structures of TbrPDEB1 of compounds 3 and 4.

The construct of the TbrPDEB1 catalytic domain expressed for use in crystallization studies contained residues 576-918. The N-terminal residues 1-575 containing two GAF domains were excluded to simplify crystallization and because the catalytic domain shows similar activity to the full protein (Chapter 3). The catalytic domain was expressed and purified...
from *Escherichia coli*. Both co-crystallization and soaking experiments were performed to generate crystals. Four TbrPDEB1 crystal structures were resolved containing, 3 (2.3 Å by soaking), 4 (2.4 Å by soaking), 8 (2.6 Å by co-crystallization) and 9 (2.6 Å by soaking). Details of the crystals and resolved structures are provided in Table 1.

**Table 1. Statistics on diffraction data and structural refinement.**

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*Crystallography data courtesy of Erin.S. Balasubramaniam and David G. Brown University of Kent*

*Overall (last shell: 3: 2.46 - 2.4 Å; 4: 2.37 - 2.31 Å; 9: 2.63 - 2.56 Å).*
The crystallographic asymmetric unit of the unliganded structure of TbrPDEB1 described in Chapter 4 contained two molecules, one of which was involved in crystal lattice contacts at the entry of the TbrPDEB1 substrate binding pocket. This phenomenon has continued in the liganded structures of TbrPDEB1, they show similar lattice contacts in one of the two molecules of the crystal lattice. In the structures of 3, 4 and 9 both molecules in the asymmetric unit contain the inhibitors and the molecules not showing crystal lattice contacts are discussed here (Chain A). In the structure of 8 only the molecule showing crystal lattice contacts in the asymmetric unit contains the inhibitor, therefore this chain will be discussed (Chain B). The conformations of Chain A are similar across the four liganded and the unliganded TbrPDEB1 structures (mean RMSD: 0.3 Å), similarly Chain B is similar (mean RMSD: 0.4 Å), however Chain A and Chain B consistently differ to a greater extent (mean RMSD: 0.6 Å).

**Figure 2**: X-ray crystal structures of 3 (A) and 4 (B) bound to TbrPDEB1. A gray surface indicates the protein surface in the region of the P-pocket. The 2D structures of the compounds are shown to the bottom right of the figures.

The crystal structures of 3 and 4 presented here show a highly conserved conservation and reveal a probable reason for the observed lack in selectivity. Although docking studies suggested 3 and 4 fit well in the P-pocket, both compounds are missing the P-pocket. Instead, the flexible tails of these compounds fold back on themselves in a hydrophobic collapse and the tetrazole head group forms π-π interactions with the F880 in the HC2 pocket far from the P-pocket (Figure 2). The fact that 2, 3, 4 and many analogues of these compounds fail to show selectivity for TbrPDEB1 suggests that compounds which
effectively address the P-pocket may not have been found yet and addressing the P-pocket in an energetically favorable way may be challenging. To overcome this challenge and obtain significant selectivity for TbrPDEB1 over human PDE4, we hypothesized that increased rigidity of the scaffold might be needed to force occupation of the P-pocket by the side chain.

5.3 Structural analysis of PDEs

The systematic approach to the analysis of the PDE catalytic domain crystal structures described in Chapter 2 allowed the creation of the PDEStrIAn database, a tool to support the design of novel PDE inhibitors. Although no previous crystal structures contain motifs interacting with the P-pockets of parasite PDEs, the information on structural elements addressing the Q2 pocket from which the P-pocket extends can be used to support the design of such compounds. The data in PDEStrIAn covers the 168 PDE crystal structures across 10 PDE families (1-10) and two parasite PDE families (B and C) published in the Protein Databank at the time of writing. A disparity in the number of crystallization studies across the PDE families is evident, with many PDE subtypes yet to be crystalized and several PDE subtypes only crystalized once or twice (Figure 3A). This disparity has consequences for the consistency of the comparison, as a lack of structural data on certain PDE subtypes prevents a full understanding of selectivity across all PDE families. Of equal importance is the range of co-crystallized ligands. Without diverse structural motifs explaining the SAR of inhibitors for each PDE family, the drivers of selectivity across PDE subtypes can only be partially understood. Despite these limitations, the PDEStrIAn database provides the most complete overview of PDE crystal structures to date and a useful toolbox for the design of ligands targeting the P-pocket.

To build the PDEStrIAn database, the canonical sequence of each PDE subtype was aligned to create a master sequence alignment and this was used to superpose the crystal structures. The highly conserved fold across the PDE families is evident when structures are overlaid (Figure 3B). Using the alignment, a consistent binding site was defined and a novel nomenclature for regions of the pocket and pocket residues was devised (Figure 3C). Three regions surround the conserved glutamine residue Q874 (Q, Q1 and Q2), three regions
surround the hydrophobic clamp between F877 and V840 (HC, HC1 and HC2), three regions surround the metal ion binding region (MB, MB1 and MB2) and the final region of the pocket covers a solvent exposed area (S). The division of the pocket into 10 regions allows a detailed discussion of the pocket regions addressed by PDE inhibitors.

The protein-ligand interactions in each PDE crystal structure were encoded in IFP (interaction fingerprint) bit strings to compare structures and identify scaffolds which interact with a specific region of the pocket. Aggregating the results by the pocket regions shows the Q region to engage in extensive hydrogen bonding, the HC region to involve extensive aromatic interactions and the HC2 and MB2 regions to form exclusively hydrophobic interactions, with the other pocket regions showing mixed sets of interactions (Figure 3D). These results are restricted to the compounds that have been crystalized to date and do not include the interactions formed in the crystal structures presented here. A set of two examples of scaffolds are shown for each pocket region in Figure 3E, these form interactions characteristic of the scaffolds which interact with the regions.
Figure 3: A) Number of crystal structures in the Protein Databank of each crystalized PDE subtype. B) Overlay of all PDE crystal structures in the Protein Databank shown as backbone ribbons. C) The PDE substrate binding pocket (PDE4D, PDB code 1OYN) showing regions of the pocket color coded. D) The frequency of interaction types found in each region of the PDE substrate pocket. The interaction types are coded by color as described below the graph. E) Two examples of scaffolds that interact with each region of the pocket.

5.4 P-pocket vector

The parasite specific P-pocket can be seen as an extension of the Q2 pocket found in all PDE structures. We reasoned that the identification of inhibitors that address the Q2 pocket could perhaps support the design of ligands that can address the P-pocket. Ligands forming
interactions with the Q2 pocket were identified using IFPs from the human PDE crystal structures. Specifically the Q2 pocket residues that are aligned with the P-pocket residues in TbrPDEB1, T841, Y845, N867, M868, E869 and L870. This resulted in 88 structures, of which 51 only form a hydrophobic interaction with T841 and were discarded. The remaining 37 structures were composed of PDE5, PDE9 and PDE10 structures with inhibitors bound.

A P-pocket vector was defined in the TbrPDEB1 crystal structure containing compound 4. The P-pocket vector is equivalent to vector 10 as described in the scaffold analysis presented in Chapter 2, page 62. The trajectory was drawn between the center of the catechol ring in 4 and the β-carbon of M868 at the far side of the P-pocket. Using superposed crystal structures this vector was compared to the binding modes of the ligands and the vector they take towards the Q2 pocket. This comparison showed that ligands interacting with the Q2 pocket in PDE5 (10, Figure 4A) and PDE9 (11, Figure 4B) do not follow the P-pocket vector. However, ligands interacting with PDE10 (12, Figure 4C) follow the P-pocket vector closely and stick deep into the Q2-pocket. The three linked ring systems in 12 enforce a linear trajectory. Docking studies (unpublished data) suggested that in TbrPDEB1 12 would position the 1-hydroxy-2-naphthonitrile group in the P-pocket. However this group was not found to form strong interactions with the P-pocket residues when docked. The biphenyl scaffold of 12 is also found in the PDE4 inhibitor 13 (Figure 4D). Although shorter and not extending deep into the Q2 pocket, the biphenyl scaffold of 13 does follow the same P-pocket vector as 12, with the exception of the meta positioned nitro group. As an inhibitor of PDE4, 13 forms a highly relevant reference molecule. The subnanomolar potency of 13 against PDE4D indicates that the biphenyl scaffold should not impede potency in TbrPDEB1 and a series of TbrPDEB1 inhibitors rigidified with biphenyl scaffolds were planned.24
Figure 4: Examples of compounds that interact with Q2 pocket residues that are aligned with the P-pocket residues. In A (1XOZ22) 10 (tadalafil, blue) is bound to PDE5A (green) forming hydrophobic interactions with A783, F787 and I813 in the Q2 pocket. In B (3JSI25) 11 (WTC, orange) is bound to PDE9A (beige) forming hydrophobic interactions with L481 and V507 in the Q2 pocket. In C (4AEL26) 12 (AZ5, green) is bound to PDE10A (blue) forming both a hydrogen bond and π – π interaction with Y683 and hydrophobic interactions with E711 and V712. In D (3IAD24) 13 (D159153, purple) is bound to PDE4D (cyan) forming no specific interactions with the Q2 pocket, only hydrophobic interactions. The interactions between ligands and the P-pocket residues and key residues of the binding site are shown in an IFP diagram. In the IFP figure each residue is described by 5 bits representing interaction types according to their position and color (see legend in figure). The red arrow shows the vector from the center of the ring of 4 towards the β-carbon of M868, indicating the desired trajectory for a ligand to interact with the P-pocket.

5.5 Towards selective TbrPDEB1 inhibitors via rigidification
Superposing 13 with 3 (Figure 5A) and 4 (Figure 5B) shows that a biphenyl scaffold covers the catechol core of the pyrazolinones. Substituting the catechol for the biphenyl scaffold found in 13 provides a rigid scaffold from which to address the P-pocket without the risk of hydrophobic collapse shifting the vector out of the pocket. The nitro group in 13 was not included in the fragment merging due to metabolism concerns and because the meta orientation of the group would not follow the P-pocket vector. The first rigidified inhibitor of TbrPDEB1 was the bare phenyl 14, showing a significant loss in affinity compared to 3 (Figure 5A). However, the selectivity of 14 for PDE4B is just 3 fold, down from 10 fold in the case of 3. Introducing an amide to the para position and replacing the iso-propyl with a cycloheptyl improves activity and brings the PDE4B selectivity below 2 fold (15). Since the phthalazinone 2 is more active than the pyrazolinones 3 and 4, a series of phthalazinone analogues were made to explore interacting with the P-pocket.

The first of the rigidified phthalazinones had a nitrile groups positioned towards the P-pocket (Figure 5C, 16). This improved activity, but the nitrile has a tenfold selectivity for PDE4B. Replacing the nitrile with a carboxylic acid maintained potency while making the compound equipotent for both TbrPDEB1 and PDE4B (17). The phthalazinone analogue of 15 with an amide oriented towards the P-pocket, improved potency slightly, but did not affect selectivity (18). The para substituent was further expanded with a tetrazole resulting in a 10 fold selectivity for TbrPDEB1 (19). An ethyl alcohol on the 3 position of the tetrazole removed selectivity, but maintained TbrPDEB1 activity (8). Adding a second amide to 16 resulted in the most potent of the compounds in the series, with an IC$_{50}$ of 0.032 µM and a 13 fold selectivity for TbrPDEB1 (9). Both 8 and 9 were crystalized bound to TbrPDEB1 and the structures clearly show the ligands occupying the P-pocket providing clear evidence that targeting the P-pocket can result in selective compounds. Both compounds form hydrogen bonds with residues in the P-pocket further stabilizing the binding. In the crystal structure of 8 (Figure 5D), the ethyl alcohol extends from the tetrazole to form a hydrogen bond with the backbone carbonyl of E869. In the crystal structure of 9 (Figure 5E) the second amide enters the P-pocket and forms hydrogen bonds with the backbone carbonyls of both M868 and E869.
Figure 5: A) Overlay of the crystal structure of 3 (cyan) bound to TbrPDEB1 (yellow) and 12 (green) showing the P-pocket vector in red. B) Overlay of the crystal structure of 4 (cyan) bound to TbrPDEB1 (yellow) and 12 (green) showing the P-pocket vector in red. C) Rigidification of 3 and 4 through scaffold hopping to the biphenyl scaffold of 12 and the
SAR around extension from the 4 position on the phenyl pointing to the P-pocket. D) The crystal structure of 8 (cyan) bound to TbrPDEB1 (yellow) showing the P-pocket vector in red. E) The crystal structure of 9 (cyan) bound to TbrPDEB1 (yellow) showing the P-pocket vector in red. An IFP sequence is shown below A, B, D and E which describes interactions between the ligands and key pocket residues and the P-pocket residues (a description of IFPs is provided below Figure 4).

5.6 Hitting the P-pocket

The rigidification of pyrazolinone and phthalazinone TbrPDEB1 inhibitors to enforce interaction with the P-pocket has a significant effect on selectivity and in contrast to the flexible ligands 3 and 4, these ligands are interacting with the P-pocket. However, even these rigidified inhibitors of TbrPDEB1 show variable selectivity, the short hydrogen and, nitrile, carboxylic acid or amide extensions of 14 - 18 do not show selectivity for TbrPDEB1. This can be expected since 13 is a potent PDE4 inhibitor, however the change in selectivity seen even with small substituents is significant. This indicates that sidechain movements required to fit the para substituted biphenyl series incur an energy penalty in PDE4 binding. This can be exploited by extending further into the P-pocket by for example substituting the amide of 18 for a tetrazole, a change that provides 19 with a 10 fold selectivity for TbrPDEB1. However, extended rigidified molecules are still required to fit the P-pocket tightly, as the extension of 19 with an ethyl alcohol (8) loses selectivity. This indicates that PDE4 is able to bind rigidified compounds and the selectivity for TbrPDEB1 is easily lost through the placement of functional groups that are better accommodated in PDE4 through induced fit.

The crystal structure binding mode of 9 bound to TbrPDEB1 shows the formamido-acetamide tail enters the P-pocket forming hydrogen bonds with backbone carbonyl oxygen atoms of M868 and E869 (Figure 7), rather than with the amino acid side chains. The formation of the dual hydrogen bonds provides a stronger P-pocket interaction than is formed by 8 and the formamido-acetamide tail is also more rigid than the ethyl alcohol of 8. Together these factors may explain the greater potency and selectivity of 9. The density in the crystal structure of 9 bound to TbrPDEB1 is lowest in the region of the formamido-acetamide tail, indicating movement of this group in the crystal. The density of the M-loop
which interacts with the formamido-acetamide tail is also low. Together this indicates that
the binding of 9 is dynamic in this region and multiple conformations and hydrogen bonding
patterns may be present in situ. Given the flexible nature of the M-loop, such dynamic
binding may be an advantage when targeting the TbrPDEB1 P-pocket.

Addressing the P-pocket has proven to be a successful method to induce selectivity for
TbrPDEB1. The crystal structures presented here provide insight into the lack of selectivity
observed in previous TbrPDB1 inhibitors and show how selectivity can be achieved by
addressing the P-pocket. Through rigidification compounds can be forced to occupy the P-
pocket. The formation of additional interactions with P-pocket residues is key to achieving
potent and selective compounds. Drug discovery efforts targeting TbrPDEB1 for
controlling HAT will benefit from the availability of the first ligand bound crystal structures
of TbrPDEB1 and the first potent and selective TbrPDEB1 inhibitor.

5.7 Experimentals
Subcloning, expression, purification and crystallization of catalytic domain tbrPDEB1. The catalytic domain of TbrPDEB1 with amino acids 576-918 was amplified by PCR and subcloned into the expression vector pET28a. The resultant plasmid pET-PDEB1 was transferred into E. coli strain BL21 (Codonplus) for overexpression. The E. coli cell carrying pET-PDEB1 was grown in LB medium at 37°C to absorption of A600 = 0.7 and then 0.1 mM isopropyl β-D-thiogalactopyranoside was added to induce the overexpression at 15°C for 24 hours. Recombinant PDEB1 was passed through a Ni-NTA column (Qiagen), subjected to the thrombin cleavage, and further purified by the columns of Q-Sepharose and Sephacryl S300 (Amersham Biosciences). A typical purification yielded over 10 mg TbrPDEB1 with a purity >95% from a 2-liter cell culture.

Crystallization, structure determination and analysis of TbrPDEB1. The catalytic domain of the TbrPDEB1 (576-918) was crystallized by vapor diffusion against a well buffer of 14-24% PEG3350, 0.4 M Na formate, 0.2 M guanidine chloride, 0.1 M MES pH 6.5 at 4°C, 10mg/mL TbrPDEB1 (0.3mM) and 20% ethylene glycol. A soaking ratio of 1:1 and co-crystallization ratio of 1 protein:1.7 ligand was used for crystallization. Diffraction data was collected at DIAMOND on beamlines 104-1, 102, 104. The crystal of the unliganded The crystals were of space group C2 with cell dimensions of a = 111.7, b = 115.3, c = 68.2 Å, and β = 108.3° and contained two molecules in the AU. The structures were solved by molecular replacement program AMoRe, using the TbrPDEB1 structure as the initial model. The structure was rebuilt by the program O and refined by the program CNS to R-factor/R-free of 0.205/0.224. Secondary structure determination was performed using DSSP. Sequence alignment of the TbrPDEB1, LmjPDEB1, TcrPDEC and hPDE4B catalytic domains was performed using ClustalW 2.1. Structural superposition of the Cα atoms of TbrPDEB1 (4I15, chain A) with LmjPDEB1 (2R8Q, chain A), TcrPDEC (3V94, chain A) and hPDE4B (1XM4, chain B) was performed with MOE (Molecular Operating Environment version 2011.10). All structures were visualized using Pymol 1.5.0.3.

Inhibition assay. The scintillation proximity assay (SPA) described by De Koning et al., was followed exactly for the determination of full length PDE activities. The assay was used to determine IC₅₀ values of purchased compounds on TbrPDEB1 and hPDE4B using
a cAMP substrate concentration of 0.5 μM. The PDE was collected by sonification of supernatants of PDE overexpressing Sf21 cells. The PDE activity of the enzyme was determined in at least duplicates by published procedures.\textsuperscript{37,38} Enzyme concentrations were set so that <20\% of the cAMP was consumed during the assay. Blank values were measured in the presence of denatured protein and always resulted in <2\% of the total radioactivity.

**Synthesis**

**General Information.** Chemicals and reagents were purchased from commercial suppliers and were used without further purification. Microwave reactions were performed with Biotage Initiator single mode cavity, producing controlled irradiation at 2450 MHz in sealed reaction vials (capable of witholding elevated pressure) and with magnetic stirring. For column chromatography commercially available Silica Gel 60 (particle size 0.040–0.063 mm) was used. Gradient flash column chromatography purification was performed on Biotage Isolera with prepacked silica (KP-sil) cartridges supplied by Biotage and ethylacetate in n-heptane as eluent, unless otherwise stated. Analytical thin layer chromatography was performed using glass sheets precoated with Silica Gel 60 F\textsubscript{254} and visualization of components was made by UV (254 nm), I\textsubscript{2} and/or Pancaldi’s solution followed by heating. Analytical HPLC–MS was performed on a Shimadzu LC-20AD liquid chromatography pump system with a Shimadzu LCMS-2010 liquid chromatography mass spectrometer equipped with an electrospray ion source using caffeine as reference. The purity for all compounds was established to be ≥95\% by HP-LC, \textsuperscript{1}H NMR and \textsuperscript{13}C NMR, unless otherwise stated. The preparation of the bromine precursors of 13 and 14 has been described.\textsuperscript{14}
1-isopropyl-3-(6-methoxy-[1,1'-biphenyl]-3-yl)-4,4-dimethyl-1H-pyrazol-5(4H)-one (14). To a 2-5 ml microwave vial with 104 mg 3-(3-Bromo-4-methoxyphenyl)-1-isopropyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (0.291 mmol), phenylboronic acid (41 mg, 0.336 mmol), Pd(PPh₃)₂Cl₂ (23 mg, 0.033 mmol), Et₃N (64 µl, 46.5 mg, 0.459 mmol) and 2.0 ml EtOH were added. The mixture was irradiated for 35 minutes at 120°C, then without further washing absorbed onto KP-Sil 15g column and flash chromatography was performed with a 6-65% EtOAc in n-heptane gradient over 18 column volumes. The product 13 was obtained as white crystals, in 62 mg with 98% purity (0.181 mmol, 69% yield). ¹H-NMR: (250 MHz, CDCl₃) δ 7.86 – 7.78 (m, 2H), 7.62 – 7.36 (m, 5H), 7.06 (d, J = 8.6, 1H), 4.55 (hept, J = 6.9, 1H), 3.90 (s, 3H), 1.60 (s, 1H), 1.51 (s, 6H), 1.41 (d, J = 6.7, 6H).

5'-(1-cycloheptyl-4,4-dimethyl-5-oxo-4,5-dihydro-1H-pyrazol-3-yl)-2'-methoxy-[1,1'-biphenyl]-4-carboxamide (15). To a 0.5 mL microwave vial with 3-(3-Bromo-4-methoxyphenyl)-1-cycloheptyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (197 mg, 0.50 mmol) suspended in EtOH (1.25 mL), was added [4-(N,N-dimethylaminocarbonyl)phenyl]boronic acid (90 mg, 0.55 mmol), triethylamine (174 mL, 1.25 mmol) and Pd(PPh₃)₃Cl₂ (109 mg, 0.2 mmol). The mixture was irradiated for 20 minutes at 120°C. The mixture was fixed onto a KP-Sil 25g column and flash chromatography was performed with a 5-75% EtOAc in n-heptane gradient over 20 column volumes. The product 14 was isolated as an off-white solid with >95% purity (180 mg, 86% yield). ¹H NMR (400 MHz, CDCl₃) d (ppm) 7.89 (d, J = 8.4 Hz, 2H), 7.80 (d, J = 2.2 Hz, 1H), 7.76 (dd, J = 8.6, 2.3 Hz, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.7 Hz, 1H), 6.10 (br s, 1H), 5.79 (s, 1H), 4.44 - 4.17 (m, 1H), 3.86 (br s, 3H), 2.06 - 1.72 (m, 6H), 1.47 (s, 6H), 1.71 - 1.49 (m, 6H).

Synthetic route of 8, 9, 16 - 21
The phthalazinone compounds (8, 9, 16-22) was synthesized as racemes of the r, s, or s, r diastereomers.

rac-(1S,6R)-6-(3-bromo-4-methoxybenzoyl)cyclohex-3-ene carboxylic acid (20). AlCl3 (31.6 g, 237 mmol) was dissolved in DCM (370 ml) and cooled to -78°C. 1-bromo-2-methoxybenzene (36.9 g, 197 mmol) in DCM (37 ml) was added to the solution dropwise. 3a,4,7,7a-tetrahydroisobenzofuran-1,3-dione (30 g, 197 mmol) dissolved in DCM (50 ml) was added dropwise. The reaction was stirred at -78°C for 30 min and 5 hours at rt. The reaction mixture was poured on ice cold water and filtered over a glass filter, yielding a white solid. This was packed on silica with DCM/MeOH/acetic acid solution and purified.
on a gravity column with 1:1:0.05 EtOAc:n-heptane:acetic acid (3 column volumes) and then 3:1:0.05 EtOAc:n-heptane:acetic acid (3 column volumes), 9:5:0.5 DCM:MeOH (4 column volumes), 9:1 DCM:MeOH (4 column volumes) and 1:1 DCM:MeOH (2 column volumes), yielding 17 as a white solid with >95% purity (12 g, 18% yield). \(^1\)H NMR (300 MHz, DMSO): \(\delta\) 12.14 (br s, 1H), 8.04 (d, J=1.8 Hz, 1H), 7.95 (dd, J=6.6 Hz, J=1.9 Hz, 1H), 7.21 (d, J=8.4 Hz, 1H), 5.62 (dd, J=10.0 Hz, J=24 Hz, 2H), 4.18-3.96 (m, 1H), 3.94 (s, 3H), 2.91-2.85 (m, 1H), 2.61-2.23 (m, 4H).

**rac-**\((4aR,8aS)-4-(3-bromo-4-methoxyphenyl)-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (21).** rac-\((1S,6R)-6-(3-bromo-4-methoxybenzoyl)cyclohex-3-ene-carboxylic acid (7.0 g, 20.64 mmol, 17) and hydrazine (1.22 ml, 24.77 mmol) were dissolved in ethanol (90 ml) and flushed thoroughly with argon. After 6 hours of refluxing, the reaction mixture was cooled to rt and filtered over a glass filter and washed with ethanol, yielding 18 as a white solid with >95% purity (5.4 g, 78% yield). \(^1\)H NMR (300 MHz, CDCl3): \(\delta\) 8.51 (s, 1H), 7.99 (d, J=2.1 Hz, 1H), 7.69 (dd, J=8.7 Hz, J=2.4 Hz, 1H), 6.92 (d, J=8.7 Hz, 1H), 5.81-5.69 (m, 2H), 3.94 (s, 3H), 3.38-3.30 (m, 1H), 3.04-2.96 (m, 1H), 2.86-2.82 (m, 1H), 2.26-2.19 (m, 3H).

**rac-**\((4aR,8aS)-4-(3-bromo-4-methoxyphenyl)-2-cycloheptyl-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (22).** Sodium hydride (1.96 g, 48.9 mmol) was added to a solution of rac-\((4aR,8aS)-4-(3-bromo-4-methoxyphenyl)-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (8.2 g, 24.46 mmol, 18) in dry N,N-dimethylformamide keeping the mixture below 25ºC. Bromocycloheptane (6.77 ml, 48.9 mmol) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was poured on cold water and extracted with EtOAc. The collected organic layers were washed with water and brine, dried on Na2SO4 and filtered. The solvent was partially removed under reduced pressure, n-heptane was added and a white solid precipitated. The mixture was filtered off over a glass filter and the residue was washed with n-heptane yielding the product 19 as a white solid with >95% purity (7.66 g, 73% yield). \(^1\)H NMR (300 MHz, CDCl3): \(\delta\) 7.99 (d, J= 2.1 Hz, 1H), 7.72 (dd, J= 8.7 Hz, J= 2.1 Hz, 1H), 6.92 (d, J= 8.7 Hz, 1H), 5.81-5.56 (m, 2H), 4.84-4.75 (m, 1H), 3.94 (s, 3H), 3.28-3.20 (m, 1H), 3.04-2.96 (m, 1H), 2.71(t, J= 4.9 Hz, 1H), 2.24-1.52 (m, 15H).
rac-5'-(4aS,8aR)-3-cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxybiphenyl-4-carbonitrile (16). rac-(4aR,8aS)-4-(3-bromo-4-methoxyphenyl)-2-cycloheptyl-4a,5,8,8a-tetrahydropthalazin-1(2H)-one (1 g, 2.318 mmol, 19), Na₂CO₃ (3.48 ml, 6.95 mmol, 2M (aq)) and 4-cyanophenylboronic acid (0.511 g, 3.48 mmol) were dissolved in 1,2-dimethoxyethane (12 ml) and degassed with argon. PdCl₂(dppf) (0.085 g, 0.116 mmol) was added to the reaction mixture and degassed again before irradiating at 110°C for 60 minutes. 1,2-Dimethoxyethane was removed under reduced pressure. The remaining water layer was neutralized with saturated NH₄Cl (aq) and extracted with DCM. The organic layer was washed with water and evaporated under reduced pressure. Purification was carried out with a normal phase chromatography with DCM:MeOH (0.2:2:2% 1-10-2 columns), yielding 20 as a white solid with >95% purity (0.85 g, 81% yield). ¹H NMR (300 MHz, DMSO): δ 7.94–7.88 (m, 3H), 7.76–7.71 (m, 3H), 7.26 (d, J = 7.5 Hz, 1H), 5.65 (m, 2H), 4.67 (app br s, 1H), 3.84 (s, 3H), 3.53–3.46 (m, 1H), 2.81–2.72 (m, 2H), 2.17–2.12 (m, 2H), 1.97–1.49 (m, 13H).

rac-5'-(cis)-3-cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxy-[1,1'-biphenyl]-4-carboxylic acid (17). To a solution of rac-(cis)-4-(3-bromo-4-methoxyphenyl)-2-cycloheptyl-4a,5,8,8a-tetrahydropthalazin-1(2H)-one (2.59 g, 6.0 mmol, 19) and 4-carboxyphenyl boronic acid (1.1 g, 6.6 mmol) in DME (33 mL), aqueous Na₂CO₃ (2M, 9.0 mL, 18.0 mmol) was added. The mixture was degassed with argon for 15 minutes, PdCl₂dppf (0.22 g, 0.30 mmol) was added and the mixture was stirred at 90°C for 19 hours. The mixture was cooled to rt, sat. aq. NH₄Cl was added the water layer was extracted with EtOAc. The combined organic layers were washed with water, brine, dried with Na₂SO₄ and concentrated to afford 21 as a brown solid with >95% purity (2.72 g, 96% yield): ¹H NMR (300 MHz, DMSO-d6) δ (ppm) 12.95 (s, 1H), 8.01 (d, J = 8.1 Hz, 2H), 7.92 (dd, J = 8.7, 2.3 Hz, 1H), 7.77 (d, J = 2.3 Hz, 1H), 7.65 (d, J = 8.2 Hz, 2H), 7.25 (d, J = 8.9 Hz, 1H), 5.72 – 5.59 (m, 2H), 4.71 – 4.65 (m, 1H), 3.84 (s, 3H), 3.53 – 3.46 (m, 1H), 2.80 – 2.72 (m, 2H), 2.17 – 2.11 (m, 2H), 1.94 – 1.36 (m, 13H).

rac-5'-(cis)-3-cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxy-[1,1'-biphenyl]-4-carboxamide (18). Through a solution of rac-(cis)-4-(3-
bromo-4-methoxyphenyl)-2-cycloheptyl-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (100 mg, 0.23 mmol), (4-aminocarbonylphenyl) boronic acid (42 mg, 0.26 mmol) and Na₂CO₃ (74 mg, 0.70 mmol) in DME (2 mL) and water (0.5 mL), nitrogen was bubbled for 5 minutes. Then, PdCl₂(PPh₃)₂ (8.1 mg, 0.012 mmol) was added and the mixture was stirred at 100°C for 20 hours. The mixture was cooled to rt, water was added and the water layer was extracted three times with EtOAc. The combined organic layers were washed with brine, dried with Na₂SO₄ and concentrated. The crude product was purified with flash chromatography (eluent Heptane/EtOAc) to afford 15 as a white solid with >95% purity (53 mg, 44% yield): ¹H NMR (400 MHz, CDCl₃) δ (ppm) 7.89 (d, J = 6.2 Hz, 2H), 7.82 (dd, J = 6.5, 1.7 Hz, 1H), 7.76 (d, J = 1.7 Hz, 1H), 7.63 (d, J = 6.2 Hz, 2H), 7.04 (d, J = 6.5 Hz, 1H), 6.12 (br s, 1H), 5.81 – 5.52 (m, 3H), 4.87 – 4.77 (m, 1H), 3.87 (s, 3H), 3.34 – 3.28 (m, 1H), 3.05 – 2.98 (m, 1H), 2.15 – 2.10 (m, 2H), 2.09 – 1.94 (m, 2H), 1.93 – 1.84 (m, 1H), 1.83 – 1.40 (m, 10H).

rac-(4aR,8aS)-2-cycloheptyl-4-(6-methoxy-4'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-3-yl)-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (19). A mixture of 5'-3-(cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxy-[1,1'-biphenyl]-4-carbonitrile (701 mg, 1.545 mmol), sodium azide (1095 mg, 16.85 mmol) and ammonium chloride (839 mg, 15.69 mmol) in DMF (15 mL) was irradiated at 150°C for 75 min. The reaction mixture was diluted in EtOAc and washed with brine acidified with HCl. The organic phase was then evaporated under reduced pressure yielding 16 as a white powder with >95% purity (728 mg, 95% yield). ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.62 (s 1H), 7.61 (d, J = 8.2 Hz, 2H), 7.40 (dd, J = 8.7, 2.4 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.7 Hz, 1H), 5.40 (dd, J = 42.9, 9.0 Hz, 2H), 4.27 – 4.33 (m, 1H), 3.43 (s, 3H), 2.95 – 3.00 (m, 1H), 2.44 (d, J = 17.9 Hz, 1H), 2.33 (t, J = 6.0 Hz, 1H), 1.78 (t, J = 20.3 Hz, 2H), 1.51 – 1.61 (m, 2H), 1.28 – 1.45 (m, 5H), 1.02 – 1.22 (m, 6H).

rac-(4aR,8aS)-2-cycloheptyl-4-(4'-(2-(2-hydroxyethyl)-2H-tetrazol-5-yl)-6-methoxy-[1,1'-biphenyl]-3-yl)-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (8). A mixture of 2-cycloheptyl-4-(6-methoxy-4'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-3-yl)-4a,5,8,8a-tetrahydrophthalazin-1(2H)-one (99.7 mg, 0.201 mmol), 2-bromoethanol (0.090 ml, 1.205 mmol) in DMF (2 mL) was heated to 60°C for 3 hours. The reaction mixture was
mixed with EtOAc and washed with water. The organic phase was evaporated under reduced pressure and placed on a (10 g flash) silica column and flash chromatography was performed with a 0-85% EtOAc in n-heptane gradient. The product was obtained as a 4:1 mixture of 8 with the (1-hydroxyethyl) isomer as a white powder with >95% purity (28 mg, 26% yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ (ppm) 8.20 (d, $J = 7.2$ Hz, 2H), 8.14 (dd, $J = 8.8$, 2.3 Hz, 2H), 7.68 (d, $J = 7.5$ Hz, 2H), 7.05 (d, $J = 8.5$ Hz, 1H), 5.73 (dd, $J = 55.1$, 10.4 Hz, 2H), 4.84 (t, $J = 4.6$ Hz, 2H), 4.77 – 4.82 (m, 1H), 4.23 – 4.28 (m, 2H), 3.88 (s, 3H), 3.30 – 3.36 (m, 1H), 3.01 (d, $J = 16.1$ Hz, 1H), 2.76 (t, $J = 5.9$ Hz, 1H), 2.49 (br s, 1H), 2.15 – 2.24 (m, 2H), 1.96 – 2.10 (m, 2H), 1.85 – 1.93 (m, 1H), 1.70 – 1.81 (m, 3H), 1.45 – 1.66 (m, 7H).

rac-N-(2-amino-2-oxoethyl)-5'-(cis)-3-cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxy-[1,1'-biphenyl]-4-carboxamide (9). To a solution of rac-5'-(cis)-3-cycloheptyl-4-oxo-3,4,4a,5,8,8a-hexahydrophthalazin-1-yl)-2'-methoxy-[1,1'-biphenyl]-4-carboxylic acid (300 mg, 0.64 mmol), DIPEA (0.11 mL, 0.64 mmol) and glycaminide hydrochloride (84 mg, 0.76 mmol) in DCM (14 mL) at 0°C, EDCI (146 mg, 0.76 mmol) and HOAt (86 mg, 0.64 mmol) were added. After stirring at rt for 2.5 hours, additional glycaminide hydrochloride (70 mg, 0.64 mmol) and DIPEA (0.11 mL, 0.64 mmol) were added and the mixture was stirred for 20 hours. The reaction mixture was poured out onto 0.5 M HCl and extracted three times with DCM. The combined organic layers were washed with water, brine, dried with Na$_2$SO$_4$ and concentrated. The crude product was purified with flash chromatography (eluent DCM/acetonitrile) to afford 9 as a white solid with >95% purity (202 mg, 57% yield): $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ (ppm) 8.69 (t, $J = 5.9$ Hz, 1H), 7.95 (d, $J = 8.5$ Hz, 2H), 7.90 (dd, $J = 8.7$, 2.3 Hz, 1H), 7.78 (d, $J = 2.3$ Hz, 1H), 7.61 (d, $J = 8.5$ Hz, 2H), 7.37 (s, 1H), 7.24 (d, $J = 8.9$ Hz, 1H), 7.04 (s, 1H), 5.72 – 5.61 (m, 2H), 4.70 – 4.64 (m, 1H), 3.85 – 3.80 (m, 5H), 3.53 – 3.46 (m, 1H), 2.81 – 2.72 (m, 2H), 2.19 – 2.11 (m, 2H), 1.94 – 1.41 (m, 13H).

References

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36. The PyMOL Molecular Graphics System. version 1.5.0.3 edn (Schrödinger, LLC, New York, NY, U.S.A.).
