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Catechol Pyrazolinones as Trypanocidals: Fragment-Based Design, Synthesis and Pharmacological Evaluation of Nanomolar Inhibitors of Trypanosomal Phosphodiesterase B1

Kristina M Orrling,[1] Chimed Jansen,[1] Xuan Lan Vu,[2] Vreni Balmer,[3] Patrick Bregy,[2†] Anitha Shanmugham,[4] Paul England,[4] Paul Cos,[5] Louis Maes,[5] Emily Adams,[6] Erika van den Bogaart,[6] Eric Chatelain,[7] Jean-Robert Ioset,[7] Andrea van de Stolpe,[1] Stèphanie Zorg,[1] Johan Veerman,[8] Thomas Seebeck,[2] Geert Jan Sterk,[8] Iwan de Esch,[1] Rob Leurs[1]*

[1]Leiden/Amsterdam Centre of Drug Research (LACDR), Amsterdam Institute of Molecules, Medicines & Systems (AIMMS), Division of Medicinal Chemistry, VU University Amsterdam, De Boelelaan 1083,
1081 HV Amsterdam, The Netherlands,

[2]Institute of Cell Biology, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland,

[3]Institute of Parasitology of the University of Bern, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland,

[4]IOTA Pharmaceuticals, St John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, United Kingdom

[5]Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Groenenborgerlaan 171, 2020 Wilrijk, Belgium

[6]Department of Biomedical Research, Royal Tropical Institute Amsterdam, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands

[7]DND*i* (Drugs for Neglected Diseases *initiative*), 15 Chemin Louis Dunant, 1202 Geneva, Switzerland

[8]Mercachem, PO-Box 6747, 6503 GE Nijmegen, The Netherlands

*Corresponding Author E-mail: R.Leurs@vu.nl

ABSTRACT

Trypanosomal phosphodiesterases B1 and B2 (TbrPDEB1 and TbrPDEB2) play an important role in the life cycle of *Trypanosoma brucei*, the causative parasite of human African trypanosomiasis (HAT), also known as African sleeping sickness. We used homology modelling and docking studies to guide fragment growing into the parasite-specific P-pocket in the enzyme binding site. The resulting catechol pyrazolinones act as potent TbrPDEB1 inhibitors with IC_{50} values down to 49 nM. The compounds also block parasite proliferation (e.g. VUF13525 (**20b**): *T. brucei rhodesiense* IC_{50} = 60 nM, *T. brucei brucei* IC_{50} = 520 nM, *T. cruzi* = 7.6 μM), inducing a typical multiple nuclei- and kinetoplast phenotype without being generally cytotoxic. The mode of action of **20b** was investigated with recombinantly engineered trypanosomes expressing a cAMP-sensitive FRET sensor, confirming a dose-response related increase of intracellular cAMP levels in trypanosomes. Our findings further validate the TbrPDEB family as antitrypanosomal target.

INTRODUCTION

Human African trypanosomiasis (HAT), commonly known as African sleeping sickness, is an extremely deadly disease; without treatment, all patients entering second stage HAT following the spread of the parasite to the CNS will eventually die.[1] Millions of people are living in the endemic areas in rural central Africa, where poverty and poor access to accurate healthcare hamper the chances of survival of infected patients.[2, 3] Even if the number of reported cases has dropped to its lowest level in 50 years, epidemics, increased political turbulence, and resistance emergence can quickly alter this trend.[3, 4] HAT is caused by the kinetoplastid protozoa *Trypanosoma brucei* and is transmitted by infected tsetse flies (*Glossina* ssp.). Two subspecies of *T. brucei* are infectious to humans, *T. b. gambiense* and *T. b. rhodesiense*. The course of the disease, symptoms and appropriate treatment depend on the etiological subspecies.[2] Despite the high medical need, current treatment options are limited and suffer from suboptimal dosage regimens and/or severe toxicity.[2] During the second stage of the disease, the only choices are the arsenic-containing drug melarsoprol and the ornithine decarboxylase inhibitor eflornithine, used as monotherapy or in combination with nifurtimox (NECT).[5] However, eflornithine is not effective against *T. b. rhodesiense*. Consequently, public-private initiatives such as the Drugs for Neglected Disease *initiative* investigate new approaches to target HAT.[6]

The mapping of the *T. brucei* genome revealed the presence of five trypanosomal cyclic nucleotide phosphodiesterases (PDEs).[7] These enzymes play an important role in the regulation of cyclic nucleotide levels by catalysing the hydrolysis of the phosphodiester bond of cAMP and/or cGMP. In humans, 11 main families of PDEs can be distinguished, and several of the human PDE enzymes have been extensively explored as molecular targets for a diverse set of diseases conditions, e.g. COPD, erectile dysfunction, pulmonary arterial hypertension, asthma,

psoriasis, type 2 diabetes, depression, cognitive disorders, and psychosis. Although the exact role of cAMP in the lifecycle of the trypanosomes is not fully understood,[8] two trypanosomal PDEs have been found to play a pivotal role in *T. brucei* proliferation.[9, 10] Knocking down of both TbrPDEB1 and TbrPDEB2 simultaneously leads to an arrest of parasite cell division, lysis of the parasites, and to elimination of the infection *in vivo* in the infected mouse model.[11] Additional studies on a hit from a high throughput screening campaign, PPS54019 (**1**), pharmacologically validated TbrPDEB1 and TbrPDEB2 as anti-trypanosomal targets.[12] In fact, **1** was once synthesised as potent human PDE4 inhibitor.[13] The benefits of extracting acquired knowledge from very advanced drug discovery programs targeting human proteins for use in neglected diseases drug discovery cannot be overestimated. Scarcity in the drug research pipeline can thus be compensated with better pharmacological predictability and an early understanding of possible adverse effects. A recent investigation shows that the hPDE4 inhibitor piclamilast (**2**) and analogues also inhibit the TbrPDEB1 and TbrPDEB2 (Figure 1).[12, 14] Inhibition of TbrPDEB1 has furthermore been observed with analogues of tadalafil (**3**)[15] and sildenafil (**4**),[16] both hPDE5 inhibitors, however at high micromolar concentrations.

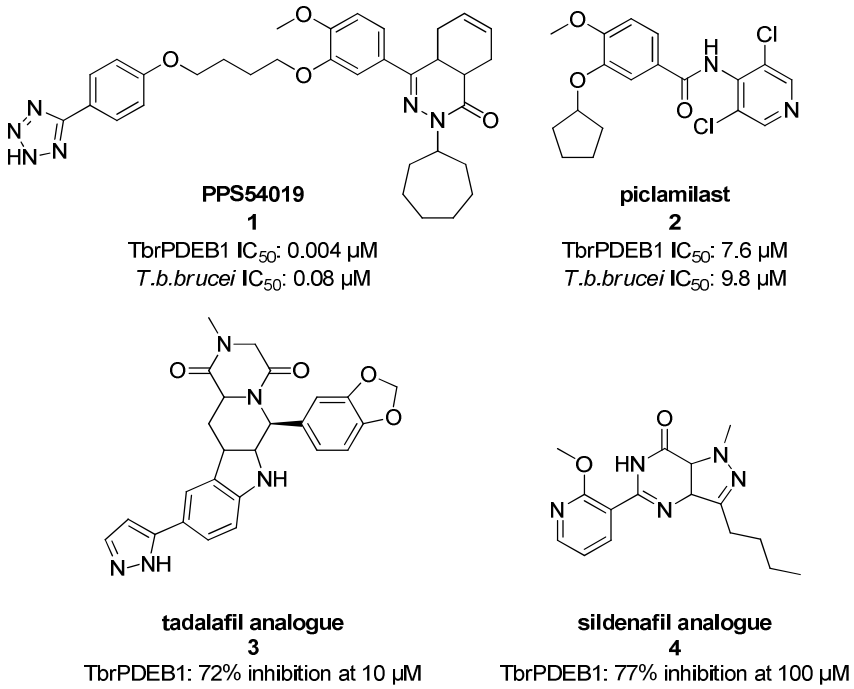


Figure 1. Known TbrPDE inhibitors, **1**,[12] **2**,[14] **3**,[15] and **4**[16].

These findings indicate that in the area of neglected infectious diseases, basing drug discovery efforts on lead compounds originally developed for human PDE targets can be quite a successful strategy. Such an approach proved not only successful in the discovery of the first TbrPDEB1 inhibitors **1**–**4**, but also the recent discovery of antiplasmodial compounds based on the PDE5 inhibitor tadalafil.[17]

Following up on chemotypes related to HTS hit **1**, catechol pyrazolinone **6a** (TbrPDEB1 inhibition with IC_{50} = 12 µM) was discovered. This fragment contains several structural features of known human PDE inhibitors and can be considered a merger of the known PDE4 inhibitor rolipram (i.e., catechol moiety connected to a cyclopentyl group, also present in **2**) and of published cardiotonic hPDE3 inhibitors **5**[18, 19] and hPDE4 inhibitors in the patent literature[20] (i.e., the pyrazolinone moiety) as outlined in Scheme 1. Following these fragment-based drug design considerations, we investigated the putative binding mode of these compounds by constructing a

homology model of TbrPDEB1 based on the x-ray structure (PDB id 2R8Q)[21] of the *Leishmania major* orthologue LmjPDEB1 (66% sequence identity). Interestingly, the so-called P-pocket, a unique sub-pocket that extends from the invariant glutamine (Gln874) through the protein to the solvent in the ligand-binding site of the LmjPDEB1 proved to be conserved in our model of TbrPDEB1.

Docking studies of rolipram suggested good accommodation in the homology model of TbrPDEB1. The docked binding poses of rolipram identified the catechol motif as the main potential contributor of affinity to the protein, being well positioned in the hydrophobic clamp between Phe877 and Val839 and hydrogen bonding with Gln874, while the lactam ring was less well positioned, making no specific interactions (Figure 2a). However, biochemical measurements indicate rolipram to be inactive at >100 μM on the TbrPDEB1 enzyme.

A pyrazolinone is a heterocycle related to the lactam ring of rolipram, but encompasses a conjugated π-system and additional possibility to interact with aromatic residues in the binding pocket (Figure 2b).

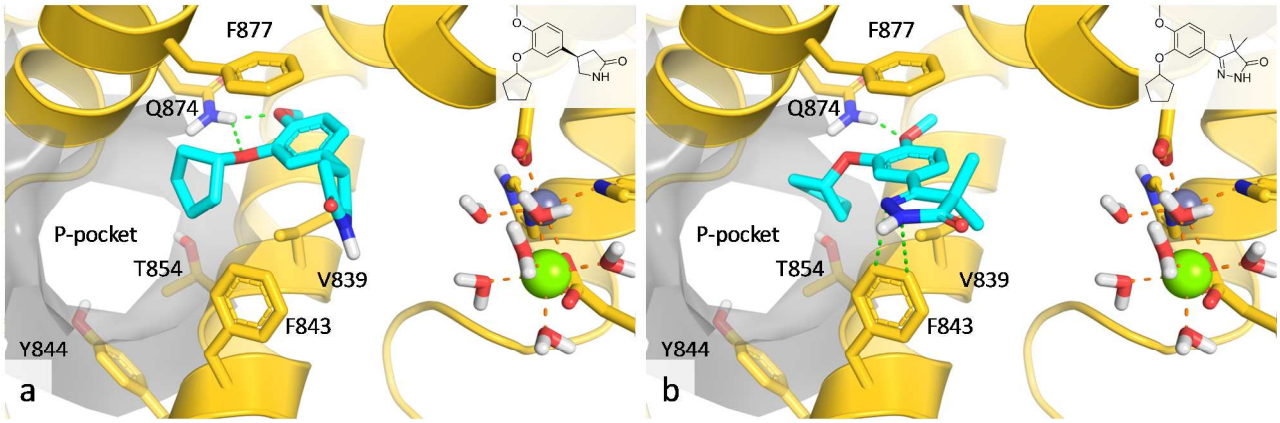
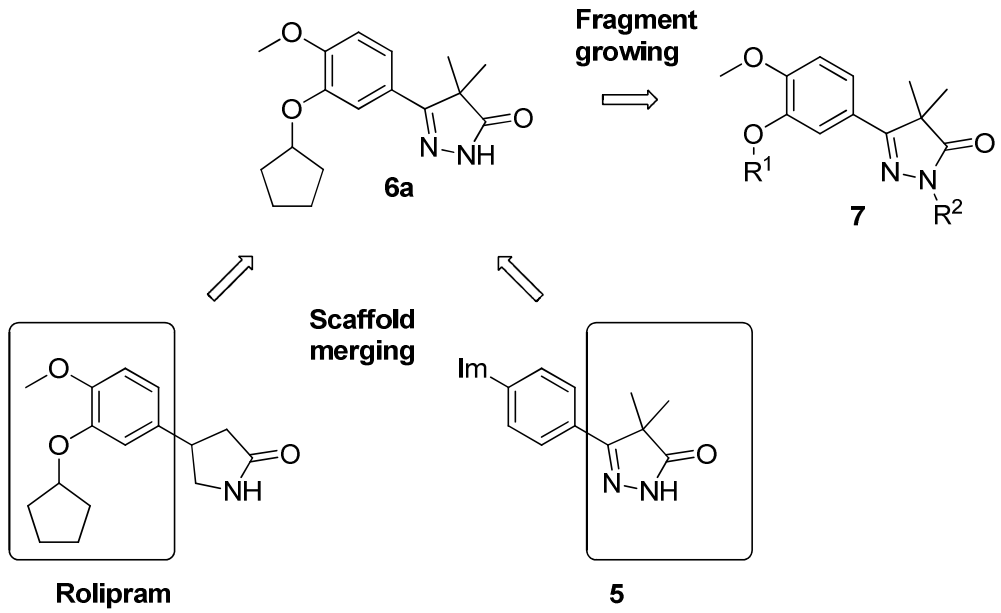


Figure 2. Proposed binding modes of (a) **rolipram** and (b) pyrazolinone **6a** in the TbrPDEB1 homology model. Both compounds interact with the highly conserved Gln874 and Phe877 and

the cyclopentyl moiety is placed at the entrance of the P-pocket. (b) The pyrazolinone ring is involved in a π– π interaction with Phe843.

In this binding pose, the cyclopentyl ring of **6a** is placed at the entrance of the P-pocket, making this an interesting region to optimize, leading to compounds with the general structure **7** (Scheme 1). A second vector to be decorated was placed at the pyrazolinone nitrogen (R^2), in line with for example phthalazinone-based hPDE4 inhibitors.[22]

Scheme 1. The design idea behind the original TbrPDEB1 inhibitor **6a**, where rolipram contributed the catechol moiety and **5**[18, 19] the pyrazolinone scaffold. The general structure of the herein reported TbrPDEB1 inhibitors is represented by **7**.



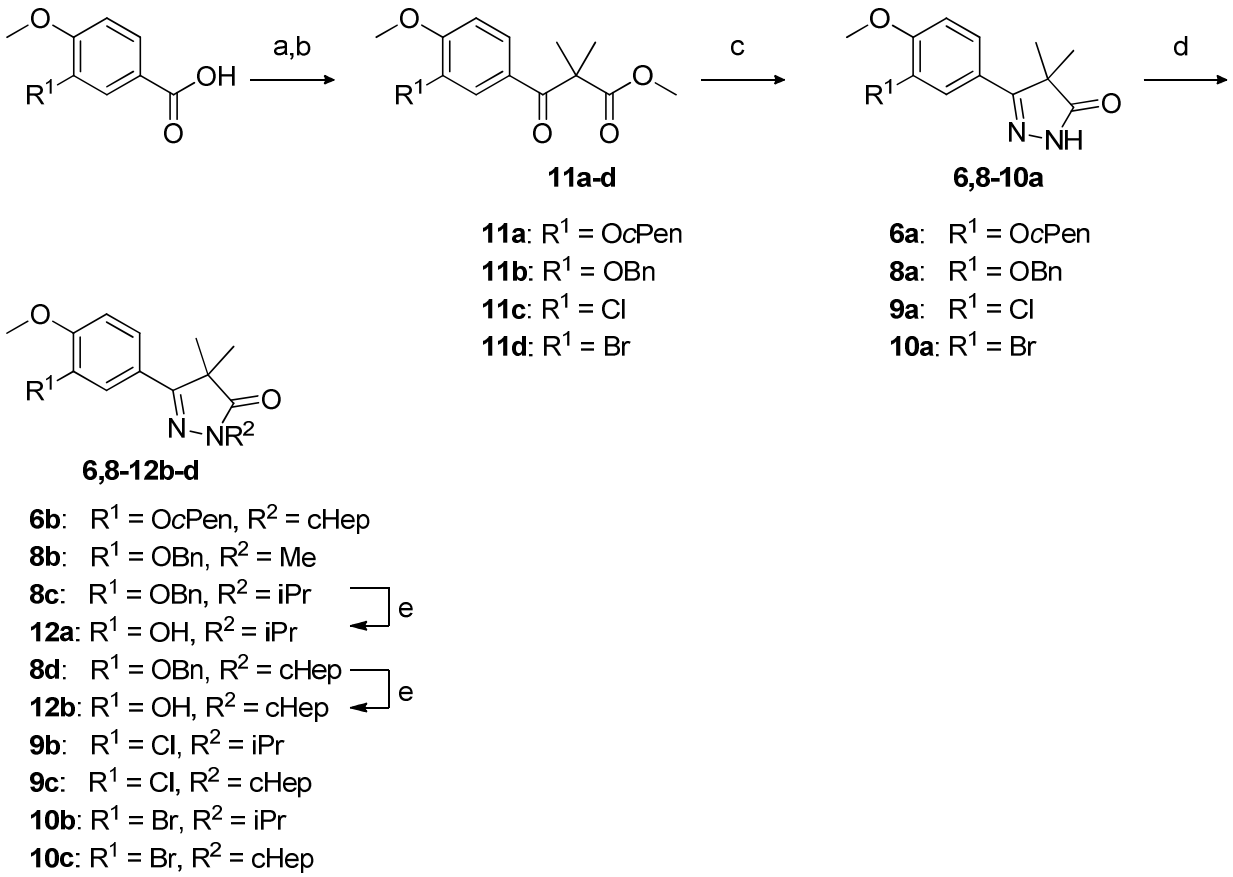
RESULTS AND DISCUSSION

Homology model of TbrPDEB1. The homology model of TbrPDEB1 was constructed based on the TbrPDEB1 sequence published by Zoraghi and Seebeck (Uniprot: Q8WQX9)[9] and the structure of LmjPDB1 (2R8Q),[21] the only orthologue of TbrPDEB1 crystallized to date. The B

chain of the LmjPDEB1 structure was used to construct the homology model, since the B-factors in the region of the catalytic site is lower in the B chain. The residues on the surface of the catalytic site show a very high sequence homology with only one V836I substitution. The P-pocket region has two substitutions N881M and R885K, both on the far side of the P-pocket from the invariant glutamine (Gln874). The overall sequence identity of the catalytic domains is 66%.

Synthetic Chemistry. The pyrazolinones **6**,**8**–**10a** were formed in good yields (67 – 99%) from a heterocyclisation reaction with aqueous hydrazine and the corresponding *β*-ketoesters **11a**–**d**.[18, 19] In contrast to previously reported synthetic routes, the *β*-ketoesters **11a**–**d** were synthesised from the corresponding benzoic acids via the benzoyl chloride and the enolate of methyl isobutyrate (Scheme 2). The ring-nitrogen was alkylated using NaH and isopropylbromide or cycloheptylbromide and heated either by microwave (MW) irradiation or refluxing overnight, depending on the reaction scale, to give **8c,d** and **9**–**10**,**b**,**c**. The methyl group in pyrazolinone **8b** was introduced in a similar fashion, but using K_2CO_3 as base. In an attempt to reduce the number of reaction steps cycloheptylhydrazine hydrogen chloride salt was used in the pyrazolinone-formation reaction, but this resulted in a complex reaction mixture and poor yield (17%) of required product **9c**. Reductive debenzylation of **8c**,**d** afforded the phenols **12a**,**b** required for further derivatization.

Scheme 2. Synthetic route to pyrazolinones[a].

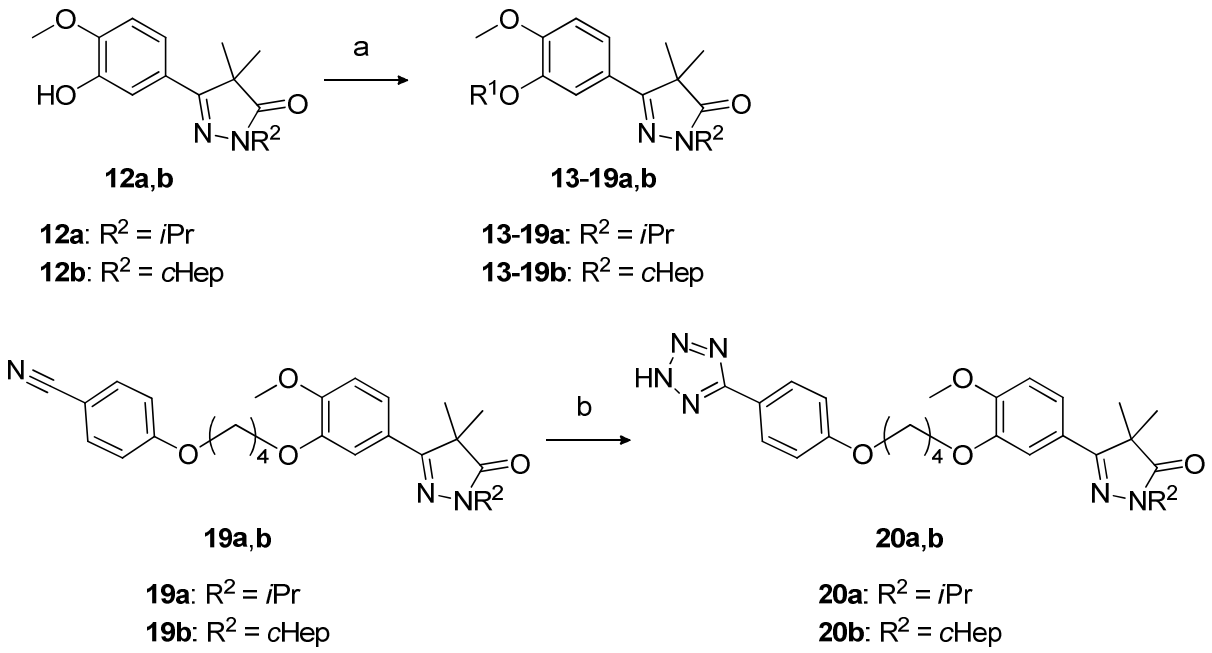


[a] Reagents: (a) $(COCl)_2$, DMF, CH_2Cl_2, 1-18 h or $SOCl_2$, rt, 1-2 h; (b) methyl isobutanoate, LDA, THF, –45 °C, 30 min, then added to acyl chloride, –55 °C to rt, 1–18 h; (c) N_2H_4, EtOH, reflux, 18 h; (d) **8b**: K_2CO_3, DMF, MW (145 °C, 20 min), then MeI, MW (145 °C, 20 min); **8c**,**d, 9–10b**,**c**: R^2Br, NaH, DMF/MeCN, MW (120 °C, 40 min); (e) Pd/C, NH_4CHO_2, MeOH:CH_2Cl_2 (2:1), 40 °C, 18 h.

A diverse set of benzyl derivatives was introduced on the phenols **12a**,**b** with either of two MW-assisted nucleophilic substitution protocols to afford inhibitors **13**–**20a**,**b** (Scheme 3, Table 2) using substituted bromides or chlorides, where chlorides normally needed longer reaction times for full conversion. For the transformation of nitriles **19a**,**b** into the corresponding tetrazoles **20a**,**b** a novel expedient MW-assisted protocol was developed (Scheme 3). Compared

to conventional refluxing overnight, the yield was improved from 19% to 62–75% and reaction time was reduced to one hour.

Scheme 3. Synthetic route to catechol pyrazolinones.[a]



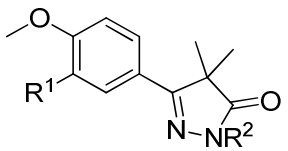
[a]Reagents: a) Method A: R^1Br, K_2CO_3, DMF/MeCN, MW (120 °C, 30 min), then R^1Br or R^1Cl, MW (120 °C, 20–60 min); method B: NaH, DMF, R^1Br, MW (120 °C, 15–30 min; b) NaN_3, NH_4Cl, DMF, MW (160 °C, 1 h).

Pharmacological measurements. All compounds were tested in at least two independent experiments for inhibition of TbrPDEB1-mediated ^{3}H-cAMP hydrolysis, using scintillation proximity assay and recombinant TbrPDEB1 expressed in Sf21 insect cells, as described previously.[12] The compounds were further tested for inhibition of *T. brucei* bloodstream form

trypomastigote proliferation and cytotoxicity on MRC5 fibroblasts using established techniques.[23, 24]

To insure a fast testing–design iteration, assessment of TbrPDEB2 inhibition was omitted at the initial screening stage, as suppression of parasite proliferation would imply concomitant inhibition of TbrPDEB1 and TbrPDEB2. In addition, two previous studies on TbrPDE inhibitors suggest high degree of equipotency against the paralogues,[12, 14] as expected from the high sequence homology. Similarly, the antiparasitic effect of the compounds was constrained to the non-infective disease model subspecies *T. brucei brucei*. The most advanced inhibitor was tested against an extended panel of PDEs, including TbrPDEB2, and trypanosome species. The inhibition of the isolated catalytic domain of TbrPDEB1 was determined using the PDELight™ HTS cAMP phosphodiesterase kit.[25, 26]

Table 1. Inhibitory potency against TbrPDEB1, *T. b. brucei* proliferation and cytotoxicity of pyrazolinones.



compd	R^1	R^2	% Yield[a]	IC_{50} (µM) TbrPDEB1	IC_{50} (µM) *T. b. brucei*	CC_{50} (µM) MRC5	LE[c]
Rolipram	–	–		>100	n.t.[b]	n.t.[b]	–
6a	O*c*Pen	H	15	12	>64	>64	0.30
6b	O*c*Pen	*c*Hep	89	0.41	>64	>64	0.30
8a	OBn	H	97	6.3	8.6	55	0.30
8b	OBn	Me	98	10	32	>64	0.27

8c	OBn	*i*Pr	73	1.0	4.5	52	0.30
8d	OBn	*c*Hep	75	0.50	6.3	>64	0.28
12a	OH	*i*Pr	96	>10	>64	>64	–
12b	OH	*c*Hep	quant	>10	8.6	>64	–
9a	-Cl	H	67	>10	>64	>64	–
9b	-Cl	*i*Pr	71	>10	>64	>64	–
9c	-Cl	*c*Hep	17	2.0	10.0	32	0.32
10a	-Br	H	87	>10	>64	>64	–
10b	-Br	*i*Pr	73	>10	>64	>64	–
10c	-Br	*c*Hep	88	2.5	34	30	0.32

[a] Isolated yield of the last step. [b] not tested. [c] ligand efficiency = (ΔG)/N, where ΔG = –RTln(IC_{50}(TbrPDEB1)) and N is the number of non-hydrogen atoms.[27]

Fragment growing.

Compared to hit structure **6a**, the benzyl analogue **8a** was slightly more potent (IC_{50} = 6.3 µM, Table 1), possibly attributed to a π – π interaction between the phenyl ring of **8a** and Phe843 in the binding pocket. By introducing aliphatic substituents (R^2) on the nitrogen atom in the pyrazolinone ring the IC_{50} values could be further improved in a size-dependent manner (R^2 = *c*Hep < *i*Pr < Me ≈ H) and submicromolar activities were observed for the cycloheptyl derivatives (e.g. **8d** IC_{50} = 0.50 µM). According to our docking studies, this gain appears to result from the hydrophobic interaction with Phe843 and slightly shifted positioning of the pyrazolinone ring strengthening the π – π interactions with Phe843 (Figure 3a). Removal of the catechol ether (**12a**,**b**) or replacing it with a chloride or bromide (**9**,**10a**–**c**) was not tolerated, although measurable potencies were observed for the two cycloheptyl derivatives **9**,**10c**. An analysis of the ligand efficiencies (LEs)[27] of this series indicates that with the 24-fold

improvement in IC_{50} values the LE values remain constant. Whereas the cyclopentyl derivatives **6a**,**b** were moderately active in the TbrPDEB1 enzyme assay, no anti-proliferative effect was observed in the parasite assay (Table 1). In contrast, the benzyloxy derivatives **8a**,**c**,**d**, all inhibited parasite proliferation with IC_{50} values < 10 µM, without being cytotoxic in MRC5 cells in this concentration range. The difference in antiproliferative efficacy might be attributed to differential parasite cell membrane permeability.

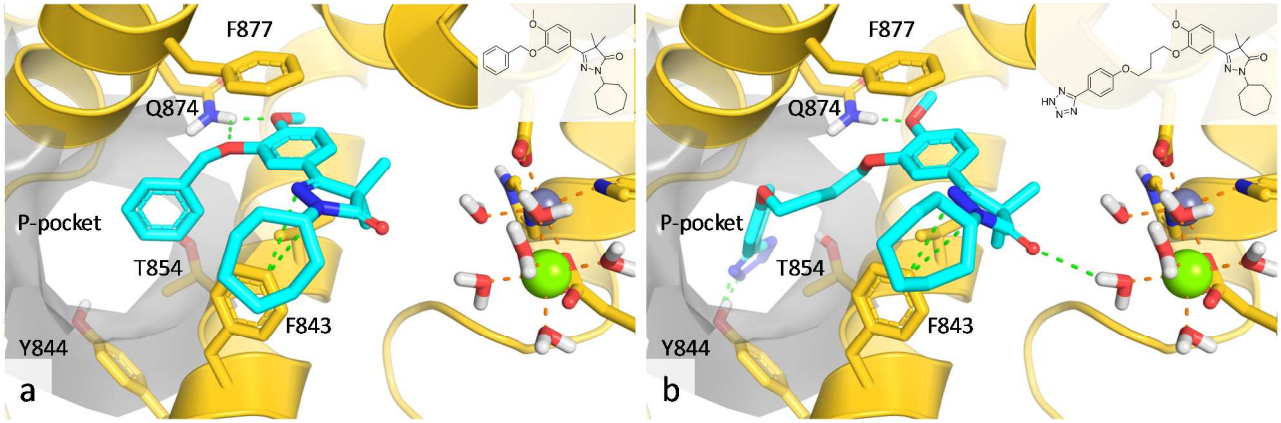


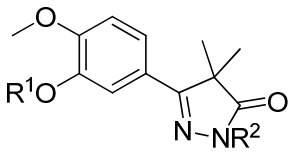
Figure 3. Proposed binding modes of a) **8d** and b) **20b** in the TbrPDEB1 homology model. The cycloheptyl rings are folded over the benzene ring of Phe843. The entrance of the P-pocket is occupied by a) the benzyl moiety of **8d** and b) the aliphatic tether of **20b** of the tetrazole, which interacts with Tyr844 further into the P-pocket.

The observed antitrypanosomal efficacy in combination with expedient synthetic access to analogues led us to proceed with the benzyl-catechol chemotype. In the docking poses, the benzene ring of these compounds is accommodated at the entrance of the P-pocket suggesting that it should be possible to grow the inhibitors further into this pocket from the 4-position, which is directed towards the P-pocket channel (Figure 3a). It was for example hypothesized that Thr854 in the P-pocket could be targeted by H-bond acceptors. We therefore synthesized the

nitriles **13a**,**b** and anisoles **14a**,**b** providing opposite electronic influences on the aromatic ring. In addition, methoxy derivatives with a two-carbon spacer (**15a**,**b**), as well as napthyl-substituted analogues **16a**,**b** were synthesized to monitor the size of the P-pocket. As the pyrazolinones with R^2 = isopropyl provided better LEs (0.30) and ligand–lipophilicity efficiencies (LLEs, Table 2, Supporting Information),[28] while good potencies were observed for R^2 = cycloheptyl in the first series, both analogues were synthesized and evaluated. As can be seen in Table 2, all analogues were active TbrPDEB1 inhibitors with IC_{50} values in the range of the parent compounds **8c**,**d**. In fact, naphthyl **16b** provided a further 2-fold increase in IC_{50} value.

The phenyl of **8c**,**d** was also replaced with *ortho*-pyridine (**17a**,**b**) and 5-methylisoxazol (**18a**,**b**) rings in order to potentially pick up an additional hydrogen bond with the second hydrogen of the Gln874 nitrogen, uniquely accessed from the P-pocket. However, this approach did not result in an increase in activity (Table 2). Interestingly, as in the previously identified TbrPDEB1 inhibitor **1**, a butyloxy spacer could be incorporated to tether the benzonitriles (**19a**,**b**) without major loss of activity; in fact, the cycloheptyl derivative **19b** was even more potent than **13b** with an IC_{50} value of 0.16 µM (Table 2).

Table 2. Inhibitory potency against TbrPDEB1, *T. b. brucei* proliferation and cytotoxicity of catechol pyrazolinones.



7-12a,b

R^2			*i*Pr					*c*Hep		
		IC_{50}		CC_{50} (µM)	LE		IC_{50}		CC_{50} (µM)	LE^a

R[1]	compd	(μM) TbrPDEB1	*T. b. brucei*	MRC5		compd	(μM) TbrPDEB1	*T. b. brucei*	MRC5	
4-CN-benzyl	**13a**	2.0	>64	>64	0.27	**13b**	1.0	9.5	>64	0.25
4-MeO-benzyl	**14a**	2.0	32	>64	0.27	**14b**	0.79	10	>64	0.25
4-MeO-phenethyl	**15a**	0.83	32	48.1	0.28	**15b**	0.63	8.1	>64	0.25
1-naphthylmethyl	**16a**	0.79	9.6	>64	0.27	**16b**	0.25	9.6	>64	0.26
2-pyridinmethyl	**17a**	2.5	35	>64	0.28	**17b**	0.50	2.5	>64	0.28
3-(5-methylisoxazolyl)-methyl	**18a**	5.0	33	>64	0.27	**18b**	0.63	8.4	33	0.27
4-(4-cyano-phenyloxy)butyl	**19a**	5.0	9.1	>64	0.22	**19b**	0.16	2.3	>64	0.25
4-(4-tetrazole-phenyloxy)butyl	**20a**	0.40	2.0	>64	0.24	**20b**	0.049	0.52	>64	0.25

[a] ligand efficiency = (ΔG)/N, where ΔG = –RTln(IC_{50}(TbrPDEB1)) and N is the number of non-hydrogen atoms.[27]

Inspired by the structure of the very potent tetrazole-encompassing tetrahydrophthalazinone **1**,[12] nitriles **19a**,**b** were converted to the corresponding tetrazoles **20a**,**b**. This transformation afforded **20b** (VUF13525), which rewardingly proved to be an efficacious inhibitor of TbrPDEB1 (IC_{50} = 49 nM) (Table 2). Docking studies of **20b** indicated that the increase in affinity could be attributed to interactions with residues in the P-pocket (Figure 3b). The proposed conformation of the catechol group is similar to the one of rolipram in its hPDE4 cocrystals[29, 30] and shows that the extended alkyl chain is able to reach and enter the P-pocket. This potentially displaces water from the length of the P-pocket and places the tetrazole at the solvent-exposed exit of the pore. In addition, the tetrazole may also form a hydrogen bond with

Tyr844, further stabilizing the conformation. Both suggestions may explain the high activity of **20b** and indicate that targeting the P-pocket may be an effective strategy for the design of potent TbrPDEB1 inhibitors.

The antitrypanosomal potency did not strictly follow the same trend as the TbrPDEB1 inhibitory activities in this series of analogues (Table 2 and Chart 1, Supporting Information). The addition of lipophilic substituents on the benzyl group R^1 often led to low IC_{50} values in the enzymatic assay, but not in the parasitic assay (c.f. **13a** and **16b**, Table 2). Moreover, we are also aware of the high cLogP[31] values (Table 1, Supporting information) of many of the cycloheptyl derivatives, accompanied with reduced aqueous solubility (visually observation), which may have been troubling the whole cell parasite assays to some extent. Yet, the most potent TbrPDEB1 inhibitor **20b**, carrying a solubilising charged tetrazole group, concentration-dependently reduced proliferation of *T. b. brucei* in the cellular assay at submicromolar concentrations (IC_{50} = 520 nM, Table 2). Compound **20b** was therefore analysed in a broader set of assays (Table 3). As could be expected on the basis of the high homology (88% sequence identity) between the catalytic domains of the TbrPDEB1 and B2 isoenzymes, **20b** also acted as a potent TbrPDEB2 inhibitor (IC_{50} = 72 nM). Similarly, the inhibition of the isolated TbrPDEB1 catalytic domain using the PDE light assay[25, 26] remained invariant (IC_{50} = 55 nM), suggesting that **20b** is not binding to any allosteric sites such as the GAF domains. To evaluate the selectivity across the PDE families, **20b** was also tested against a panel of other PDE enzymes (Table 3). The *L. major* orthologue LmjPDEB1 was inhibited with a slightly lower potency (IC_{50} = 0.13 μM). On the other hand **20b** was more potent to inhibit representative isoenzymes of PDE4A–D families (IC_{50} at 0.0012 – 0.010 μM). The relatively low bias for hPDE4 inhibition (4 – 35-fold), compared to, for example, piclamilast **2** (>1000-fold), might be attributed to the

proposed interactions with the parasite-specific P-pocket. Nevertheless, the data indicates that the binding mode of the compounds in TbrPDEB1 and hPDE4 still needs careful considerations. Evidently, the flexibility of the ligands and/or the hPDE4 protein allows accommodation in the binding site. Considering that the clinical development of hPDE4 inhibitors has been accompanied by emesis and adverse gastrointestinal and pro-inflammatory side effects,[32, 33] the level of hPDE4 inhibition must be considered. The selectivity ratio needed for a safe PDE-targeting trypanocidal is difficult to predict at this stage. The narrow therapeutic window of hPDE4 inhibitors that have advanced to clinical trials indicates that at least equipotency is preferred and most likely future lead optimization efforts should focus on a 10–100-fold selectivity for the parasite PDE.

Interestingly, besides being a good inhibitor of *T. b. brucei* proliferation, **20b** was also an excellent inhibitor of *T. b. rhodesiense* (IC_{50} = 60 nM, Table 3), the causative agent of acute East-African HAT, for which the only treatment is the arsenic-containing drug melarsoprol, associated with fatal encephalopathy in 5-10% patients.[34] Moreover, **20b** also showed some activity against *T. cruzi*, the causative agent of Chagas disease.[35] Pyrazolinone **20b** was well-tolerated by the human fibroblast cell line (CC_{50} > 64 μM) and murine macrophages, providing a sensitivity index of at least 250-fold for *T. b. rhodesiense*, i.e. well within the conventional thresholds.[36]

Table 3. Pharmacological profiling of pyrazolinone **20b**.

Enzymatic assay	IC_{50} (μM)	Cellular assay	IC_{50} (μM)
TbrPDEB1	0.049	*T. b. brucei*	0.52
TbrPDEB2	0.072	*T. b. rhodesiense*	0.06

TbrPDEB1 catalytic domain	0.055	*T. cruzi*	7.6
LmjPDEB1	0.13	MRC5	>64[a]
hPDE4A1	0.0021		
hPDE4B1	0.0046		
hPDE4C1	0.010		
hPDE4D3	0.0012		

[a] Mouse macrophage cytotoxicity was observed at 64 µM.

To confirm that the trypanocidal effect of **20b** acts via PDE inhibition, the accumulation of intracellular trypanosomal cAMP was monitored using a FRET-based cAMP sensor that was recombinantly expressed in procyclic trypanosomes. As can be seen in Figure 4a, administration of **20b** to the parasites induced a rapid and dose-dependent increase in intracellular cAMP levels, implying fast penetration over the cellular membrane. When wild-type blood-form *T. b. brucei* were incubated with **20b** for 12 hours they display the typical phenotype, associated with duplicate or multiple nuclei and kinetoplasts (Figure 4b–d), which has previously been observed in genetic[11] and chemical[12] interference of TbrPDE function. The occurrence of aberrant forms of parasites increased with the doses of **20b** administrated. After 48 hours incubation with 10 µM **20b**, parasite cells had lysed confirming the cidal effect of prolonged exposure of TbrPDEB family inhibitors.

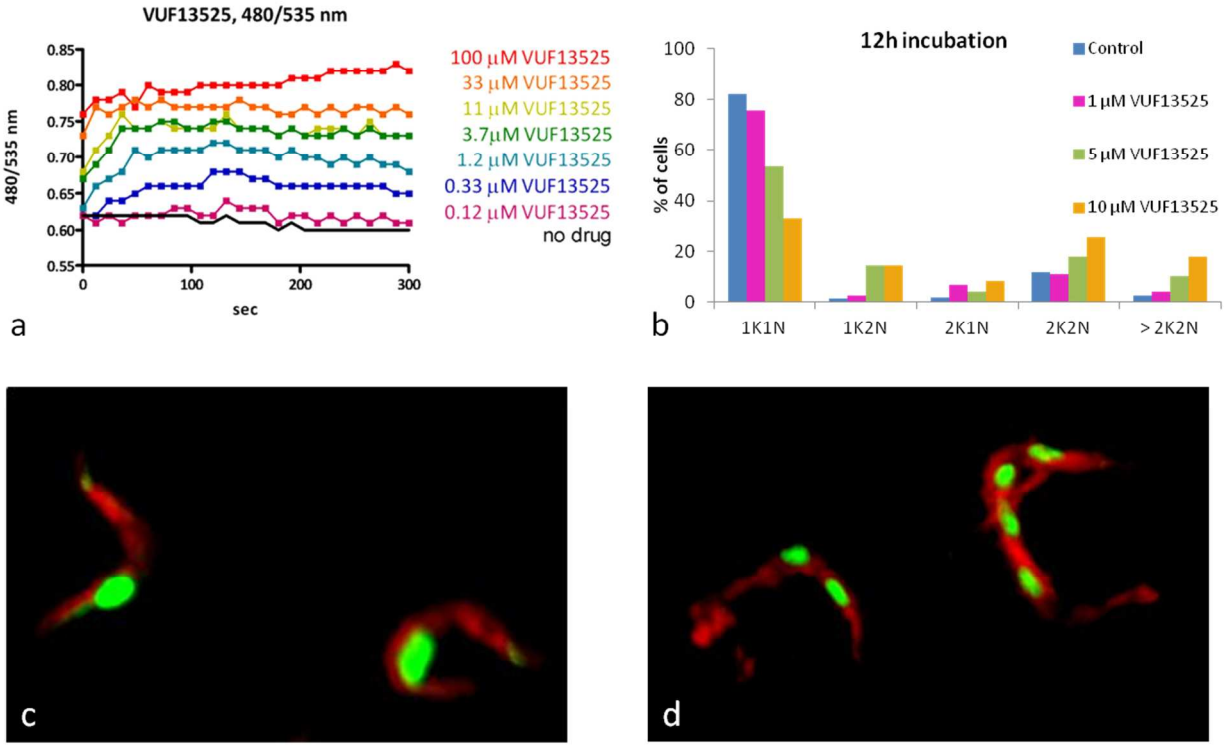


Figure 4. (a) Time-response curves after administration of TbrPDEB1 inhibitor **20b** to engineered *T. brucei* expressing cAMP-FRET sensors. (b) Occurrence of different *T. b. brucei* phenotypes after 12 h incubation with **20b**; 1N1K (1 nucleus + 1 kinetoplast); 1N2K (1 nucleus + 2 kinetoplasts); 2N1K (2 nuclei + 1 kinetoplast) and >2N2K (aberrant forms with more than 2 nuclei and/or 2 kinetoplasts). Microscope images of (c) control *T. b. brucei* 1N1K parasites and (d) *T. b. brucei* parasites after 12 h incubation with 10 µM pyrazolinone **20b** displaying (left) duplicate nuclei and (right) multiple nuclei and kinetoplasts.

CONCLUSIONS

With this study it has been shown that P-pocket directed design of TbrPDEB1 inhibitors is a successful strategy to find highly potent antitrypanosomal compounds. Structure-based and ligand efficiency-led design resulted in the cathecol pyrazolinone (**20b**) with nanomolar potencies against both TbrPDEB1 and TBrPDEB2 (49 nM and 72 nM, respectively). It also

shows excellent *in vitro* activity against *T. b. brucei* and, clinically more relevant, *T. b. rhodesiense* (60 nM), which is the causative agent of the acute East-African form of HAT. At prolonged exposure, the inhibitor also has a cidal effect on the parasites. On the contrary, mammalian cell lines seem to tolerate the catechol pyrazolinone chemotype very well; fibroblast toxicity was only observed for a handful of compounds.

For the first time, the increase in cAMP levels in the parasites upon administration of PDE inhibitor could be followed in real-time using a FRET-based cAMP sensor expressed in engineered trypanosomes. Being aware of the many challenges that remain, such as selectivity over hPDE4, blood-brain barrier penetration, acceptable pharmacokinetic profile and metabolic stability, we still believe that the promising pharmacological profile of **20b** brings new hope to the prospect of developing a less toxic alternative to melarsoprol.

EXPERIMENTAL SECTION

Homology model of TbrPDEB1.

The homology model was constructed using the TbrPDEB1 sequence published by Zoraghi and Seebeck (Uniprot: Q8WQX9).[9] The closest homologue of TbrPDEB1 crystallized to date is Leishmania major PDEB1, the structure is published as 2R8Q on the RCSB Protein Data Bank. Aligning the sequences using ClustalW 2.1[37] gave a sequence identity of 66%. The residues on the surface of the catalytic site show an even higher homology with only one substitution (V836I). The P-pocket region has two substitutions, N881M and R885K, both on the far side of the P-pocket from the invariant glutamine (G874).

The homology model was built using the Molecular Operating Environment (MOE) 2010.10 software package.[38] The B chain of the LmjPDEB1 structure was used to construct the homology

model, since the B chain showed lower B factors in the region of the catalytic site. The LmjPDEB1 structure was opened in MOE and all solvent and metal atoms were removed. The TbrPDEB1 sequence was added and the sequence alignment from ClustalW 2.1 applied. Those residues which were identical in both sequences had their atoms tethered at 200 in the 2R8Q.B model. The Amber99 forcefield and born solvation model were used during the homology model construction. There were 10 main-chain and 1 side-chain models formed, the intermediate models were refined to an RMS of 0.1 and the final model was refined once more to an RMS of 0.5. Models were protonated using the Protonate 3D function prior to refinement and scored using the GB/VI scoring function. The final model had the metal ions and the six metal-coordinating water molecules included directly from the LmjPDEB1 model, with the hydrogen atoms minimized following inclusion.

The full-length amino acid sequences of the TbrPDEB1 and TbrPDEB2 (Uniprot: Q38F42) catalytic domains were aligned using ClustalW 2.1 giving a sequence identity of 75%. Restricting the sequence alignment to the 334 residues of the catalytic domain increased the sequence identity to 88%.

Inhibitor docking.

The structures of the ligands were prepared in MOE and exported as an SDF database. A single low energy conformation was generated for each structure using Omega 2.4.3, OpenEye Scientific Software.[39] The mol2 file generated by Omega was used as input for GOLD.[40] The compounds were docked into the homology model of TbrPDEB1 described above, which was used as prepared. GOLD uses a genetic algorithm to efficiently explore the conformational space of the ligand in the protein pocket. The parameters used in GOLD include, autoscaling of operations per ligand (dependent on the number of rotatable bonds), PLP scoring function, water

included with spin, metal ions included, and the generation of 30 solutions per ligand with an RMS tolerance of 1.5 Å. Results were analyzed using MOE and binding poses were rendered in PyMOL.[41]

Synthetic Chemistry.

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 withholding 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_{254} and visualization of components was made by UV (254 nm), I_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 Xbridge (C18) 5 µm column (50 mm × 4.6 mm), using acetonitrile in 0.10% aqueous formic acid and a 5% – 90% gradient over 4.5 min followed by 2 min isocratic 90% mobile phase. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 200, 400 or 500 at 250 MHz, 400 MHz or 500 MHz, respectively. The chemical shifts for ^{1}H NMR and ^{13}C NMR were referenced to TMS via the $CDCl_3$ solvent signal (^{1}H NMR at 7.26 ppm and ^{13}C NMR at 77.16 ppm). Exact molecular masses (HRMS) were determined on Bruker micrOTOF-Q 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, ^{1}H NMR and ^{13}C NMR, unless otherwise stated. Acyl chloride precursors of β-keto esters **11a–d**[42-45] are known compounds. The synthetic preparation and spectroscopic data of catechol pyrazolinone **8a** has previously been published in a patent.[20]

Experimental details and spectroscopic data for precursors 11a,b.

Methyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)-2,2-dimethyl-3-oxopropanoate (11a).

3-(cyclopentyloxy)-4-methoxybenzoic acid (5.0 g, 21.1 mmol) was suspended in 25 mL dry DCM and DMF (3 drops) was added. Oxalyl chloride (2.78 ml, 31.7 mmol) was added dropwise under vigorous stirring and N_2 atmosphere. The reaction mixture was stirred at room temperature for 1.5 h. The reaction was monitored with TLC of small aliquots of the reaction mixture, which were diluted with MeOH and heated with a heat-gun. When full conversion was observed, the volatiles were evaporated *in vacuo* to give 3-(cyclopentyloxy)-4-methoxybenzoyl chloride as a yellow crystalline solid, which was used as such in the subsequent step without any further purification.

Lithium diisopropyl amide (13 mL, 1.8 M in THF/Hex/ether, 1.1 equiv) was added to a dry 3-neck flask with 25 mL freshly distilled THF and cooled to -45 °C and methyl isobutyrate (3.64 ml, 31.7 mmol, 1.5 equiv) was added dropwise. The mixture was stirred for 30 min at -40 °C. Freshly prepared 3-(cyclopentyloxy)-4-methoxybenzoyl chloride (approx. 21 mmol) was dissolved in dry THF (25 mL) and added dropwise during 30 min at -50 – -40 °C. The reaction mixture was stirred for another 1 h before the cooling source was removed and the stirring continued at rt overnight. The reaction mixture was acidified with 3 M HCl (aq), diluted with EtOAc (10 mL) and the aqueous layer was extracted with EtOAc (2 x 10 mL). The combined organic layers were washed with sat $NaHCO_3$ and brine, dried over $MgSO_4$ and reduced *in*

vacuo. The crude product was purified with flash chromatography to give the title compound as a pale syrup (1.1 g, 15%): ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 7.50 – 7.41 (m, 2H), 6.83 (d, *J* = 9.08 Hz, 1H), 4.82 – 4.73 (m, 1H), 3.83 (s, 3H), 3.64 (s, 3H), 2.07 – 1.94 (m, 2H), 1.94 – 1.75 (m, 4H), 1.72 – 1.59 (m, 2H), 1.54 (s, 6H); ^{13}C NMR (126 MHz, CDCl3) δ (ppm) 196.14, 176.13, 153.94, 147.59, 127.85, 122.77, 114.49, 110.64, 80.55, 56.18, 53.09, 52.66, 32.93, 24.33, 24.31; LC–MS–ESI^{+} found 321 $[M+H]^{+}$.

Methyl 3-(3-(benzyloxy)-4-methoxyphenyl)-2,2-dimethyl-3-oxopropanoate (11b). Following the procedure to **11b** using 3-(benzyloxy)-4-methoxybenzoic acid on a 148 mmol scale first gave 3-(benzyloxy)-4-methoxybenzoyl chloride as a yellow semi-solid (41.5 g, quant., >95% purity according to ^{1}H NMR); ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 7.78 (dd, *J* = 8.45, 1.94 Hz, 1H), 7.66 (d, *J* = 1.90 Hz, 1H), 7.48 (d, *J* = 7.26 Hz, 2H), 7.43 – 7.28 (m, 3H), 6.94 (d, *J* = 8.52 Hz, 1H), 5.30 (s, 1H), 5.19 (s, 3H), 3.95 (s, 3H)) and the title compound as a yellow syrup (59 g, quant., 90% purity): ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 7.55 – 7.44 (m, 4H), 7.43 – 7.26 (m, 3H), 6.88 (d, *J* = 8.4 Hz, 1H), 5.18 (s, 2H), 3.94 (s, 3H), 3.61 (s, 3H), 1.51 (s, 6H); ^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm) 195.81, 175.85, 153.58, 147.94, 136.54, 128.63, 128.05, 127.85, 127.49, 123.30, 113.78, 110.59, 70.85, 56.06, 52.97, 52.47, 24.13; LC–MS–ESI^{+} found 343 $[M+H]^{+}$.

Experimental details and spectroscopic data for pyrazolinones.

3-(3-(Cyclopentyloxy)-4-methoxyphenyl)-4,4-dimethyl-1H-pyrazol-5(4H)-one (6a). To a reaction tube with **11a** (0.851 g, 2.66 mmol), dissolved in ethanol (5.5 mL, 0.5 M reaction molarity), aqueous hydrazine hydrate (80%, 1.4 mL, 8 equiv) was added. The reaction mixture was stirred at 50 °C overnight. The reaction mixture was cooled on ice and the precipitate was filtered off over a glass filter, washed with cold ethanol and dried at 40 °C under vacuum to give

the title compound as a solid foam (0.72 g, 90%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.43 (s, 1H), 7.42 (d, *J* = 1.93 Hz, 1H), 7.27 (dd, *J* = 8.35, 2.06 Hz, 1H), 6.86 (d, *J* = 8.46 Hz, 1H), 4.92 – 4.69 (m, 1H), 3.87 (s, 3H), 2.04 – 1.74 (m, 6H), 1.71 – 1.55 (m, 2H), 1.51 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 181.71, 163.35, 151.93, 148.11, 123.93, 119.36, 112.34, 111.41, 80.68, 56.13, 47.40, 32.93, 24.21, 22.85; LC–MS–ESI$^+$ found 303, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{17}H_{23}N_2O_3$: 303.1703 found 303.1698.

1-Cycloheptyl-3-(3-(cyclopentyloxy)-4-methoxyphenyl)-4,4-dimethyl-1H-pyrazol-5(4H)-one (6b). Sodium hydride (52 mg, 1.3 mmol, 2 equiv.) was added to a chilled suspension of **6a** (195 mg, 0.65 mmol) in DMF/MeCN (2:1) under stirring and N_2 atmosphere. The reaction mixture was stirred for 15 min before bromocycloheptane (0.31 ml, 2.3 mmol, 3.5 equiv.) was added. The reaction mixture was heated at 120 °C overnight. The volatiles were evaporated *in vacuo*. The residue was diluted with EtOAc (50 mL) and washed with brined (3 x 15 mL), dried over Na_2SO_4, filtered and reduced *in vacuo*. The residue was purified via flash chromatography to give title compound as a pale solid (230 mg, 89 % yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.39 (d, *J* = 1.90 Hz, 1H), 7.28 (dd, *J* = 8.47, 1.90 Hz, 1H), 6.85 (d, *J* = 8.47 Hz, 1H), 4.91 – 4.72 (m, 1H), 4.37 – 4.18 (m, 1H), 3.86 (s, 3H), 2.03 – 1.72 (m, 12H), 1.72 – 1.47 (m, 8H), 1.43 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) 177.50, 161.48, 151.59, 147.82, 124.10, 119.35, 112.55, 111.35, 80.69, 56.07, 54.57, 48.58, 33.43, 32.95, 28.33, 24.81, 24.27, 22.86; LC–MS–ESI$^+$: m/z 399, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{24}H_{35}N_2O_3$: 399.2642 found 399.2624.

3-(3-(Benzyloxy)-4-methoxyphenyl)-4,4-dimethyl-1H-pyrazol-5(4H)-one (8a). Following the procedure to **6a** using **11b** on a 0.15 mol scale gave the title compound as a yellow solid (47.36 g. 97%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.67 (br s, 1H), 7.44 (appar. d, *J* = 7.36

Hz, 2H), 7.41 (d, *J* = 1.93 Hz, 1H), 7.39 – 7.26 (m, 4H), 6.89 (d, *J* = 8.48 Hz, 1H), 5.18 (s, 2H), 3.91 (s, 3H), 1.41 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 181.72, 163.11, 151.49, 148.35, 136.85, 128.68, 128.05, 127.39, 123.88, 119.99, 111.78, 111.38, 71.16, 56.07, 47.32, 22.62; LC–MS–ESI$^+$: m/z 325, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{19}H_{21}N_2O_3$: 325.1547 found 325.1531.

3-(3-(Benzyloxy)-4-methoxyphenyl)-1-isopropyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (8c). Following the procedure to **6b** using isopropylbromide and **8a** on a 2.46 mmol scale gave the title compound as a white solid (706 mg, 78%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.49 (d, *J* = 7.3 Hz, 2H), 7.44 (d, *J* = 1.9 Hz, 1H), 7.34 (m, 4H), 6.91 (d, *J* = 8.5 Hz, 1H), 5.22 (s, 2H), 4.50 (hept, *J* = 6.7 Hz, 1H), 3.93 (s, 3H), 1.38 (s, 6H), 1.37 (d, *J* = 7.0 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 177.85, 161.28, 151.30, 148.28, 137.00, 128.66, 128.05, 127.47, 124.11, 119.92, 112.17, 111.38, 71.36, 56.06, 48.73, 45.26, 22.67, 20.82; LC–MS–ESI$^+$: m/z 367, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{22}H_{27}N_2O_3$: 367.2016 found 367.1998.

3-(3-(Benzyloxy)-4-methoxyphenyl)-1-cycloheptyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (8d). Following the procedure to **6b** on a 1.59 mmol scale using **8a** gave the title compound as a white solid (544 mg, 81% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.49 – 7.43 (m, 2H), 7.41 (d, *J* = 2.00 Hz, 1H), 7.38 – 7.33 (m, 2H), 7.32 – 7.26 (m, 2H), 6.87 (d, *J* = 8.49 Hz, 1H), 5.20 (s, 2H), 4.36 – 4.16 (m, 1H), 3.90 (s, 3H), 1.98 – 1.74 (m, 6H), 1.67 – 1.47 (m, 6H), 1.34 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 177.41, 161.16, 151.21, 148.23, 136.98, 128.61, 127.99, 127.38, 124.09, 119.83, 112.10, 111.32, 71.31, 56.02, 54.47, 48.39, 33.39, 28.34, 24.73, 22.62; LC–MS–ESI$^+$: m/z 421, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{26}H_{33}N_2O_3$: 421.2486 found 421.2467.

3-(3-Hydroxy-4-methoxyphenyl)-1-isopropyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (12a). Ammonium formate (10 g, 159 mmol) and 10% Pd/C (50 mg, 0.047 mmol) were added to benzylether **8c** (12 g, 32.7 mmol) dissolved in MeOH/CH_2Cl_2 (2:1, 200 mL). The reaction mixture was stirred for 5 h at 40 °C. The reaction mixture was filtered through a cake of celite and the volatiles were evaporated. The residue was diluted with EtOAc, washed with brine (2 x 200 mL), dried over Na_2SO_4, filtered and reduced *in vacuo* with CH_2Cl_2 as co-solvent to give the title compound as an off-white solid (8.73 g, 96% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 2.0 Hz, 1H), 7.29 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.86 (d, *J* = 8.5 Hz, 1H), 5.97 (s, 1H), 4.49 (hept, *J* = 6.6 Hz, 1H), 3.92 (s, 3H), 1.44 (s, 6H), 1.34 (d, *J* = 6.7 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 178.06, 161.60, 148.22, 145.99, 124.91, 118.78, 112.61, 110.62, 56.10, 49.00, 45.37, 22.74, 20.85; LC–MS–ESI$^+$: m/z 277, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{15}H_{21}N_2O_3$: 277.1547 found 277.1543.

1-Cycloheptyl-3-(3-hydroxy-4-methoxyphenyl)-4,4-dimethyl-1H-pyrazol-5(4H)-one (12b). Following the procedure of **12a** using **8d** on a 1.32 mmol scale afforded the title compound as a pale oil (1.35g, quantitative yield, 99.8% purity): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.46 (d, *J* = 2.05 Hz, 1H), 7.29 (dd, *J* = 8.43, 2.08 Hz, 1H), 6.87 (d, *J* = 8.49 Hz, 1H), 6.30 (br s, 1H), 4.36 – 4.22 (m, 1H), 3.92 (s, 3H), 2.03 – 1.74 (m, 6H), 1.70 – 1.47 (m, 6H), 1.45 (s, 6H); ^{13}C NMR (101 MHz, CDCl3) δ 177.62, 161.58, 148.30, 146.00, 124.72, 118.64, 112.63, 110.63, 56.00, 54.58, 48.66, 33.37, 28.25, 24.72, 22.61; LC–MS–ESI$^+$: m/z 331, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{19}H_{27}N_2O_3$: 331.2016 found 331.1997.

General procedure to *O*-alkylations A. Corresponding phenol **12a** or **12b** was added to a MW reaction tube with K_2CO_3 (3.0 equiv) and MeCN/DMF (1:1, 0.2 M final reaction molarity). It was MW irradiated for 30 min at 120 °C. After releasing the CO_2-gas from the MW tube, the

corresponding bromide or chloride (2 equiv.) was added via a needle through the septum. The reaction mixture was again MW irradiated for 20 min at 120 °C. In case of low conversion, 1 more equivalent of halide was added and the reaction mixture was reirradiated for 40 min. The volatiles were evaporated *in vacuo*. The residue was dissolved in EtOAc and the organic layer was washed with brine and brine with sat NH_4Cl 1:1. The organic phase was dried over Na_2SO_4, filtered, and reduced *in vacuo*. The crude product was purified using gradient flash column chromatography.

General procedure to *O*-alkylations B. Sodium hydride (60% dispersed in oil, 2–5 equiv.) was added to corresponding phenol **12a** or **12b** dissolved in DMF (0.14 M reaction molarity) under N_2 atmosphere. The mixture was stirred for 5 – 15 min prior to addition of corresponding bromide (1.5 equiv). The reaction mixture was microwave irradiated at 120 °C for 15–30 min and then worked-up as in general procedure A.

4-(4-(5-(1-Isopropyl-4,4-dimethyl-5-oxo-4,5-dihydro-1H-pyrazol-3-yl)-2-methoxyphenoxy)butoxy)benzonitrile (19a). Following the general procedure **A** on a 1.12 mmol scale gave the title compound as an off-white solid (201 mg, 40%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.59 – 7.50 (m, 2H), 7.45 (d, *J* = 1.96 Hz, 1H), 7.29 – 7.23 (m, 1H), 6.96 – 6.88 (m, 2H), 6.85 (d, *J* = 8.46 Hz, 1H), 4.48 (hept, *J* = 6.70 Hz, 1H), 4.13 (dt, *J* = 14.51, 5.74 Hz, 4H), 3.85 (s, 3H), 2.08 – 2.00 (m, 4H), 1.44 (s, 6H), 1.35 (d, *J* = 6.72 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.95, 162.44, 161.51, 151.17, 148.76, 134.08, 124.27, 119.78, 119.39, 115.33, 111.13, 110.63, 103.90, 68.71, 68.13, 56.07, 48.94, 45.40, 26.14, 25.91, 22.97, 20.93; LC–MS–ESI$^+$: m/z 450, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{26}H_{32}N_3O_4$: 450.2387 found 450.2366.

4-(4-(5-(1-Cycloheptyl-4,4-dimethyl-5-oxo-4,5-dihydro-1H-pyrazol-3-yl)-2-methoxyphenoxy)butoxy)benzonitrile (19b). Following the general procedure **B** on a 0.58

mmol scale gave the title compound as an off-white solid (166 mg, 57%): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.11 (appar. d, *J* = 8.85 Hz, 2H), 7.42 (d, *J* = 1.98 Hz, 1H), 7.32 (dd, *J* = 8.43, 1.99 Hz, 1H), 7.01 (appar. d, *J* = 8.88 Hz, 2H), 6.88 (d, *J* = 8.53 Hz, 1H), 4.35 – 4.26 (m, 1H), 4.18 (t, *J* = 5.97 Hz, 2H), 4.13 (t, *J* = 5.75 Hz, 2H), 3.89 (s, 3H), 2.11 – 1.94 (m, 6H), 1.94 – 1.84 (m, 2H), 1.84 – 1.73 (m, 2H), 1.69 – 1.50 (m, 6H), 1.49 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 178.14, 175.72, 162.46, 161.57, 151.31, 148.69, 129.25, 123.92, 119.98, 116.45, 115.28, 111.31, 110.81, 68.88, 68.02, 56.12, 55.25, 49.24, 33.44, 28.21, 26.19, 26.14, 24.89, 22.88; LC–MS–ESI$^+$: m/z 504, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{30}H_{38}N_3O_4$: 504.2857 found 504.2840.

3-(3-(4-(4-(2H-tetrazole-5-yl)phenoxy)butoxy)-4-methoxyphenyl)-1-isopropyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (20a). A mixture of compound **19a** (141 mg, 0.31 mmol), sodium azide (203 mg, 3.1 mmol) and NH_4Cl (167 mg, 3.1 mmol) in DMF (6 mL) was MW irradiated at 160 °C for 1 h. The reaction mixture was cooled to RT, diluted with EtOAc, washed twice with brine acidified with HCl (aq). The first aqueous layer was extracted with EtOAc (2 x 5 mL). The combined organic layers were further washed with brine acidified with HCl (aq), reduced, and the crude product was purified with flash column chromatography (eluent: MeOH in CH_2Cl_2 with 0.5% AcOH) to afford the title compound as an off-white solid (115 mg, 75%); ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 8.11 (appar. d, *J* = 8.76 Hz, 2H), 7.44 (d, *J* = 1.71 Hz, 1H), 7.33 (dd, *J* = 8.42, 1.78 Hz, 1H), 7.02 (appar. d, *J* = 8.79 Hz, 2H), 6.89 (d, *J* = 8.49 Hz, 1H), 4.54 (hept, *J* = 6.70 Hz, 1H), 4.19 (t, *J* = 6.06 Hz, 2H), 4.14 (t, *J* = 5.77 Hz, 2H), 3.89 (s, 3H), 2.07 (dt, *J* = 12.48, 6.49 Hz, 4H), 1.49 (d, *J* = 9.55 Hz, 6H), 1.39 (d, *J* = 6.70 Hz, 6H).); MS-ESI$^+$ (m/z 493, M+H$^+$); ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm)178.53, 162.43, 161.56, 156.09, 151.29, 148.70, 129.25, 123.82, 119.92, 116.34, 115.26, 111.14, 110.61, 68.82, 67.94, 56.08, 49.50,

45.83, 26.17, 26.06, 22.90, 20.87; LC–MS–ESI$^+$: m/z 493, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{26}H_{33}N_6O_4$: 493.2558 found 493.2545.

3-(3-(4-(4-(2H-tetrazole-5-yl)phenoxy)butoxy)-4-methoxyphenyl)-1-cycloheptyl-4,4-dimethyl-1H-pyrazol-5(4H)-one (20b). Following the procedure to compound **20a** using nitrile **19b** (100 mg, 0.20 mmol) to afford the title compound as an off-white solid (95 mg, 62%): ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 8.14 (d, *J* = 8.87 Hz, 2H), 7.44 (d, *J* = 2.05 Hz, 1H), 7.30 (dd, *J* = 8.46, 2.04 Hz, 1H), 7.01 (d, *J* = 8.92 Hz, 2H), 6.88 (d, *J* = 8.53 Hz, 1H), 4.36 – 4.25 (m, 1H), 4.18 (t, *J* = 6.00 Hz, 2H), 4.13 (t, *J* = 5.94 Hz, 2H), 3.88 (s, 3H), 2.12 – 1.93 (m, 6H), 1.93 – 1.85 (m, 2H), 1.82 – 1.73 (m, 2H), 1.67 – 1.49 (m, 6H), 1.49 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) 178.14, 162.48, 161.56, 156.14, 151.29, 148.68, 129.27, 123.82, 119.93, 116.36, 115.25, 111.17, 110.66, 68.83, 67.94, 56.09, 55.26, 49.25, 33.41, 28.16, 26.18, 26.05, 24.85, 22.87; LC–MS–ESI$^+$: m/z 547, $[M+H]^+$; HRMS–ESI$^+$: $[M+H]^+$ calcd for $C_{30}H_{39}N_6O_4$: 547.3027 found 547.3015.

Pharmacological evaluation.

Expression of recombinant TbrPDEB1 and TbrPDEB2. Full length hPDE4A1, B1, C1 and D3 and TbrPDEB1 were expressed in Sf21 insect cells and full length TbrPDEB2 was expressed in yeast, as previously described.[12]

His-tagged catalytic domains of TbrPDEB1 were expressed in E. coli BL21 codon plus cells and column purified as recommended in the manual, "The Qiaexpressionist" (Qiagen). PDEs were dissolved in 10 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 5 mM $MgCl_2$.

Determination of PDE Activity. PDE activities were determined according to two different methods throughout the study.

Full length PDEs. The standard scintillation proximity assay (SPA) for determination of PDE activities was used exactly following the procedure as recently outlined by De Koning et al.,[12] (14) to analyze effects of test compounds on the enzymatic activities of TbrPDEB1, TbrPDEB2, LmjPDEB1, human PDE4A1, 4B1, 4C1 and 4D3. In these assay, the cAMP substrate concentration was 0.5 μM. Briefly, PDE activity of supernatants of sonicated PDE-overexpressing Sf21 cells was determined in at least duplicates by published procedures.[12, 46, 47] Enzyme concentrations were always adjusted so that <20% of substrate was consumed. Blank values (measured in the presence of denatured protein) were always <2% of total radioactivity.

Catalytic Domain TbrDEB1. The determination of TbrPDEB1 catalytic domain activity was performed with PDELight™ HTS cAMP phosphodiesterase kit[25, 26] in a reaction volume of 50 μL comprising 1% DMSO in a 96 well-format. PDELight assay is a non-radioactive, bioluminescent detection system for measuring the activity of PDEs developed by Lonza Rockland, Inc. This assay was used in the determination of IC_{50} values for inhibitors against catalytic domain of TbrPDEB1. This assay offers a continuous readout of luminescence based on the following reactions: PDEs hydrolyse cAMP to AMP; AMP is then converted directly to ATP by "AMP-DR", Lonza's proprietary AMP detection reagent; luciferase is then utilized to catalyze the formation of light from luceferin and the newly formed ATP. Luciferase catalysis step also regenerates AMP and therefore the light output is linear with the rate of AMP production by the PDE. The reaction uses cAMP in the concentration of 0.5 μM. From the rate of increase in light intensity over a period of time, the reaction rate is calculated. The reaction output light was monitored at 25 °C for 20 minutes using Victor2 yielding a specific activity of about 2500U/min in the given conditions. Dose-response curves were generated by plotting the percent of inhibition of PDE against the inhibitor concentrations. Nonlinear regression analysis

was used to calculate IC_{50} values from each dose-response curve by using GraphPad Prism (version 5.01).

***In vitro* susceptibility testing of trypanosomes and MRC5 fibroblasts.** *T. b. brucei* (Squib-427 strain, suramin-sensitive) or *T. b. rhodesiense* (STIB-900 strain) trypomastigotes were cultured at 37 °C and 5% CO_2 in Hirumi-9 medium,[48] supplemented with 10% fetal calf serum (FCS). Compound stock solutions were prepared in 100% DMSO at 20 mM or mg/ml. The compounds were serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

Assays were performed by adding 1.5×10^4 trypomastigotes/well containing the pre-diluted compounds. After 72 h incubation, parasite growth was assessed fluorimetrically by adding resazurin[24] for 24 h at 37 °C. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm).

For cytotoxicity evaluation, human fetal lung fibroblast were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% FCS at 37 °C and 5% CO2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the pre-diluted compounds and incubated at 37 °C and 5% CO_2 for 72 h. Cell viability was determined after addition of resazurin. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm).[23, 49]

Phenotype determination. *T. b. brucei* trypomastigotes ($1x10^6$ parasites/mL) were incubated with **20b** (1, 5, or 10 µM or vehicle) for 12 hours. Thereafter, parasites were harvested and washed once with saline. Culture aliquots were spread onto glass slides. After drying, they were fixed for 15 min with 2% formaldehyde and stained for 30 min at room temperature with Syto-9

(Invitrogen) 250 nM and Nyle Red (Sigma) 50 mg/ml. Slides were analyzed using an Olympus BX41 microscope. For each sample, >300 cells were analyzed. Cells were manually scored and assigned to the following categories: 1N1K (1 nucleus + 1 kinetoplast); 1N2K (1 nucleus + 2 kinetoplasts); 2N1K (2 nuclei + 1 kinetoplast) and >2N2K (aberrant forms with more than 2 nuclei and/or 2 kinetoplasts).

FRET cAMP sensor construct.

Expression of cAMP sensors in trypanosomes. Nikolaev *et al*.[50, 51] had developed a series of FRET-based cAMP sensors to analyze the spatial and temporal regulation of cAMP signalling in living cells. They are based on a YFP and a CFP fluorescent protein linked to various cAMP-binding domains. For use in trypanosomes, a sensor variant utilizing the cAMP binding domain of EPAC 1[52] ($K_{D(cAMP)}$ ~1 μM cAMP) was selected. For expression in procyclic trypanosomes, the EPAC 1 sensor was amplified from the corresponding pcDNA3 vector (generously provided by V. Nikolaev) using the following primers: cAMPsensor-fo (5'-TACTCGAGCTATAGGGAGACCC-AAGCTT-3'; underlined XhoI restriction site) and cAMPsensor-re (5'-TAGGATCCTA-GGTGACACTATAGATAG-3'; underlined BamHI restriction site). The PCR product was cloned into the pGAPRONEΔLII-mcs vector (pGΔLII-mcs).[53] The final construct was linearized with SpeI and transfected into the procyclic *T. brucei* strain 427[54] for stable integration into the EP procyclin locus. Transfectants were selected by adding neomycin (1μg/mL) into the medium. Expression of the EPAC 1 sensor was verified by FACS analysis and by immunofluorescence.

FRET analysis. Procyclic trypanosomes were cultured in SDM79 medium containing 5 % fetal calf serum.[55] For FRET analysis, cells were collected by centrifugation from logarithmically growing culture (50 mL), suspended in 15 ml Hanks balanced salt solution (HBSS, GIBCO),

centrifuged for 10 min at 4000 rpm and suspended to a final cell density of 5-6 x 10^7/mL in fresh HBSS buffer. Drugs were dissolved in dimethylsulfoxide (DMSO), and appropriate dilution series in DMSO were prepared. Drugs were spotted (1 μL per well) into black, flat-bottom 96-well plates, followed by the addition of 100 μL / well of HBSS and extensive mixing. Plates were allowed to equilibrate at 27 ^{o}C in the thermostatted chamber of a Gemini Spectramax fluorescence reader. Immediately before FRET recording was started, 100 μl of cell suspension was added to each well and plates were vibrated for 5 sec. Fluorescence was then measured using an excitation wavelength of 436 nm, while recording the appropriate emissions at 480 and 535 nm, with cutoff filters set at 475 and 530 nm, respectively. After finishing each series, cell motility was checked microscopically. As a positive control, a dilution series of the PDE inhibitor PPS50419[12] was included in each experiment.

ASSOCIATED CONTENT

Supporting Information. The synthetic procedures and spectroscopic data of compounds **8b**, **11c**,**d**, **9**,**10a–c**, **11c**,**d** and **13–18a**,**b**, ^{1}H NMR and ^{13}C NMR spectra of compound **20b**, a graph of the correlation between *T. b. brucei* proliferation inhibition and TbrPDEB1 inhibition of catechol pyrazolinones, and tabulated cLogP values and LLEs for all compounds.

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +31-20598-7579/7600, e-mail: R.Leurs@vu.nl

Present address

† Swiss Transfusion SRC Inc, Laupenstrasse 37, 3001 Bern, Switzerland

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ABBREVIATIONS USED

CFP **cyan fluorescent protein**

***c*Hep**	**cycloheptyl**
***c*Pen**	**cyclopentyl**
FRET	**fluorescence resonance energy transfer**
HAT	**human African trypanosomiasis**
hPDE	**human phosphodiesterase**
***i*Pr**	**isopropyl**
K	**kinetoplast**
LLE	**ligand-lipophilicity efficiency**
LmjPDEB1	***Leishmania major* phosphodiesterase B1**
MOE	**Molecular Operating Environment**
MW	**microwave**
N	**nucleus**
NECT	**nifurtimox-eflornithine combination therapy**
TbrPDEB1	***Trypanosoma brucei* phosphodiesterase B1**
TbrPDEB2	***Trypanosoma brucei* phosphodiesterase B2**
YFP	**Yellow fluorescent protein**

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