Published in final edited form as:
J Biomol Screen. 2008 August ; 13(7): 609–618. doi:10.1177/1087057108319977.

A Cell-based PDE4 Assay in 1536-well Plate format for High Throughput Screening

Steven A. Titus[1], **Xiao Li**[2], **Noel Southall**[1], **Jianming Lu**[2], **James Inglese**[1], **Michael Brasch**[2], **Christopher P. Austin**[1], and **Wei Zheng**[1]

[1]NIH Chemical Genomics Center, National Human Genome Research Institute, NIH 9800 Medical Center Drive, MSC 3370 Bethesda, MD 20892-3370

[2]BD Biosciences, 15010 Broschart Road, Rockville, Maryland 20850

Abstract

The cyclic nucleotide phosphodiesterases (PDEs) are intracellular enzymes that catalyze the hydrolysis of 3', 5'-cyclic nucleotides, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to their corresponding 5'-nucleotide monophosphates. These enzymes play an important role in controlling cellular concentrations of cyclic nucleotides and thus regulate a variety of cellular signaling events. PDEs are emerging as drug targets for several diseases including asthma, cardiovascular disease, ADHD, Parkinson's disease, and Alzheimer's disease. Though biochemical assays with purified recombinant PDE enzymes and cAMP or cGMP substrate are commonly used for compound screening, cell-based assays would provide a better assessment of compound activity in a more physiological context. Here we report the development and validation of a new cell-based PDE4 assay using a constitutively active GPCR as a driving force for cAMP production and a cyclic nucleotide gated (CNG) cation channel as a biosensor in 1536-well plates.

Keywords

phosphodiesterase; PDE IV; cyclic nucleotide gated ion channels; cell-based assay; high throughput screening

Introduction

PDEs hydrolyze 3', 5'-cyclic nucleotides including cAMP and cGMP, to their corresponding 5'-nucleotide monophosphates AMP and GMP. Both cAMP and cGMP are important second messengers coupling to the G-protein coupled receptors (GPCRs) and mediate the responses of a variety of hormones and neurotransmitters. PDEs are responsible for terminating cellular responses to hormones and neurotransmitters, which is critical for maintaining proper intracellular signaling events. There are 11 families of PDEs from 21 different genes [1,2]. Each PDE family is distinguished functionally by unique enzymatic characteristics and pharmacological profile as well as distinct tissue distribution and cellular expression patterns [3,4]. Because PDEs regulate a variety of cellular functions, they have become important drug targets for the treatment of several diseases including sexual dysfunction, asthma, chronic obstructive pulmonary disease, neurodegenerative diseases (Parkinson's disease and Alzheimer's), diabetes, vascular diseases, osteoporosis cancer and rheumatoid

Correspondence should be addressed to: Wei Zheng, Ph.D., NIH Chemical Genomics Center, National Human Genome Research Institute, NIH 9800 Medical Center Drive, MSC 3370, Bethesda, MD 20892-3370, Phone: 301-217-5733, email: wzheng@mail.nih.gov.

arthritis [2,5,6]. PDE4 isoenzymes specifically hydrolyze cAMP and are therapeutic targets for the treatment of several inflammatory disorders.

A number of biochemical assays are available for screening of PDEs which use purified recombinant enzymes and cAMP or cGMP as the substrate. However, a cell-free assay environment may not faithfully reproduce the physiological environment of the cell due to differences in buffer components, pH, co-factors, among others. In addition, compounds active in enzyme assays are often inactive in disease models due to poor cell membrane permeability, intracellular metabolism, or active sites with highly polar groups [7]. Therefore, inhibitors identified from enzyme assays usually need to be optimized in cell-based assays before proceeding to animal model studies. A cell-based PDE4 assay was described using the PDE4 gene transiently transfected into COS cells, and the PDE activity was measured with a radioimmunoassay kit in cell lysate [8]. Another cell-based assay which examined PDE5 was reported using the rat fetal lung fibroblast (RFL-6) cells and the PDE activity was measured by the scintillation proximity assay (SPA) in cellular lysates [9]. These cell-based PDE4 assays had complicated assay procedure and relatively limited screening throughput. Recently, a cell-based luciferase reporter gene assay using the cAMP responsive element (CRE) binding sequence was reported for the measurement of PDE4, PDE7 and PDE10 activities [10–12]. In general, reporter gene assays often cause shifts in measured compound activities and produces increased false positives in compound library screening due to a long cascade of signal transduction and reporter gene transcription/translation. Finally, a throughput limited cell-based PDE4 assay using a CNG cation channel as a biosensor was reported. In this assay, PDE activity was detected using the calcium dye, Fura-2, and electrophysiology (using the voltage clamp method) [13–15]. We report here the development and validation of a new high-throughput compatible PDE assay using a HEK 293 cell line co-expressing a constitutively active GPCR receptor as a driving force for cAMP production together with a CNG channel as a cAMP sensor in 1536-well plate format.

Materials and Methods

Materials

A PDE4 cell line (TSHR-CNG-HEK293), a parental cell line (CNG-HEK293) and the membrane potential dye kit were obtained from BD Biosciences (Rockville, MD). The DMEM medium, DPBS, Lipofectamine 2000, Geneticin, trypsin-EDTA, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). The fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Puromycin and the pCMV-IRES-PURO vector were obtained from Clontech (Palo Alto, CA). Forskolin, isoproterenol, and PDE inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

Establishment of stably transfected PDE4 cell line and cell culture

The cell-based PDE4 assay uses a TSHR-CNG-HEK 293 cell line (BD ACT*One*™) co-expressing a thyroid stimulating hormone receptor (TSHR) with a CNG cation channel stably expressed. This cell line was generated by transfecting 2 μg of a pCMV-TSHR-IRES-PURO plasmid with 6.25 μl Lipofectamine 2000 to the BD ACT*One*™ CNG-HEK293 cells. After 18–22 hrs at 37°C in 5 % CO_2, the transfected cells were selected in DMEM media containing 10%FBS supplemented with 250 μg/ml Geneticin and 2 μg/ml Puromycin. Selection media was changed every 3–4 days, and colonies were observed 2–3 weeks after selection. Isolated colonies were selected and transferred into 24 well-plates containing 1 ml of DMEM plus 10% FBS supplemented with 250 μg/ml Geneticin and 1 μg/ml Puromycin (as growth medium).

Cells were harvested when they reached 80–90% confluence. After detachment by Trypsin-EDTA, cells were diluted in growth medium at 7×10^5 cells/ml and 100 µl/well of cell suspension was added to Biocoat 96-well ploy-D lysine coated plates (BD Biosciences) and cultured for 16–24 hours. An equal volume of 1× ACTOne membrane potential dye solution was then added to each well and incubated for one hour. The known PDE4 inhibitor RO20-1724 was added and the fluorescence intensity which reflected PDE4 activity was measured in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA). The clones with a signal-to-basal ratio larger than 2 fold were selected as candidate cell lines for follow-up analysis. The final cell line was selected based on the IC_{50} and concentration-response values of RO 20-1724, that were closest to the values reported previously.

The TSHR-CNG-HEK293 cells were maintained in the culture medium containing DMEM, 10%FBS, 250 µg/ml Geneticin, 1 µg/ml Puromycin, 50 units/ml penicillin, and 50 µg/ml streptomycin at 37°C under a humidified atmosphere containing 5% CO_2. The parental CNG-HEK293 cells (without TSH receptor) were maintained in the same medium without Puromycin. Cells were passaged when they reached to 80 – 90 % confluence.

Cell based PDE4 assay

Cells were dispensed at a density of 1000 cells/well in black, clear bottom, tissue culture treated, 1536 well plates (Kalypsys, San Diego, CA) in 3 µl assay medium containing DMEM, 2 % FBS, 50 units/ml penicillin and 50 µg/ml streptomycin and were incubated 24 hr at 37°C with 5 % CO_2 prior to compound screening. 3 µl/well of 1 × membrane potential dye was added and incubated for 1 hr at the room temperature. The library compounds in DMSO solution or the positive control, RO 20-1724, was added at 23 nl/well with a Pintool Station (Kalypsys, San Diego, CA). After a 30-minute incubation with compounds at the room temperature, the assay plate was measured in an Envision fluorescence plate reader (PerkinElmer, Woburn, MA) in bottom reading mode with an excitation of 535 (±20) nm and emission of 590 (±20) nm. A flying reagent dispensing (FRD) workstation (Aurora Discovery, San Diego, CA) was used to dispense cells and reagents to 1536-well plates. The Kalypsys Pintool Station was used to transfer 23 nl compounds in DMSO solution to the 1536-well assay plate. The final DMSO concentration in the assay plates was under 0.5%. During library screening, all plate manipulations were done on an automated robotic system (Kalypsys, San Diego, CA).

PDE4 enzyme assay

The PDE4 A1A enzyme assay in IMAP format has been described previously [16]. Briefly, 2 µl per well of PDE4 A1A enzyme (BPS Bioscience, San Diego, CA) mixture (containing a final concentration of 0.05 ng/µl enzyme, 10 mM Tris pH 7.2, 10 mM $MgCl_2$, 1 mM DTT, 0.05% NaN_3, and 0.15% BSA) was dispensed to 1536 well black solid bottom plates. The compounds (diluted in DMSO) were added using the Kalypsys Pintool Station at 23 nl/well and then plates were spun at 1000 rpm for 15 seconds in a tabletop centrifuge. After a 5-minute incubation at room temperature, 2µl/well of 200 nM cAMP was dispensed to each well and the plates were spun at 1000 rpm for 15 seconds. The assay plates were incubated for 40 minutes at room temperature followed by an addition of 4 µl/well of IMAP detection reagent (Molecular Devices, Sunnyvale, CA). The plates were measured in a ViewLux plate reader (PerkinElmer, Woburn, MA) in fluorescence polarization detection format (Ex= 480 nm and Em = 540 nm) after one hour incubation at room temperature.

Compound Library

A collection of 2187 compounds including 1208 compounds of the Library of Pharmacologically Active Compounds (LOPAC, Sigma Aldrich) and 979 compounds from Tocris (Bristol, UK) was screened against this cell-based PDE4 assay. The compounds were

dissolved in DMSO at a concentration of 10 mM for all stock solutions. All compounds were serially diluted in DMSO at a ratio of the square root of 5 (1:2.236) to fifteen concentrations in 384-well plates. Four sets of the interplate dilution plates were subsequently reformatted into one set of 1536-well plates with concentrations ranging from 0.13 μM to 10 mM. After 23 nl compound addition, final compound concentrations in the 6 μl assay volume were from 0.49 nM to 38.3 μM.

Data analysis

The primary screening data and curve fitting were analyzed using custom software developed at the NIH Chemical Genomics Center. The maximal inhibition (100% activity) was determined by the response of 30 μM RO 20-1724 and the basal response (0% activity) was measured by the DMSO control in both the enzyme and cell-based PDE4 assays. In the counter-screen with the parental cell line, 10 μM forskolin and 30 μM RO 20-1724 were added to determine the maximal inhibition. The concentration-responses of the compounds were analyzed using the methods described in [17]. Briefly, concentration-response data were fitted to a four parameter Hill equation by minimizing the residual error between the modeled and observed responses. Concentration-response curves were divided into four classes: Class 1 contained complete concentration-response curves and r^2 values >0.9. Class 2 contained incomplete concentration-response curves lacking the lower asymptote and show r^2 values >0.9. Class 3 curves were of the lowest confidence as they were defined by a single concentration point for the inhibition. Finally, Class 4 contained all inactive compounds that did not show any concentration response curves. Curves were further subdivided based on efficacy, either >80% inhibition compared to the control as class 1.1 and partial efficacy >50% inhibition as 1.2. Clustering analysis of active compounds from the primary screening was performed with Leadscope® Hosted Client (Leadscope Inc., Columbus, OH). The results from other experiments were analyzed with Prism software (GraphPad, San Diego, CA).

Results and Discussion

This cell-based PDE4 assay uses a stably transfected CNG cation channel [14] as a biosensor whose signal can be detected by a change in membrane potential (Fig. 1). The PDE substrate, cAMP, is produced by adenylate cyclase (AC) which is stimulated by the constitutive activity of stably transfected TSH receptors coupling through the $G_{\alpha S}$ subtype of the G-proteins [18]. Previous studies have demonstrated that the TSH receptor exhibits a moderate level of constitutive activity in the absence of ligand when it is over expressed in mammalian cells [19,20]. The constitutive activity of transfected TSH receptors in the PDE4 cell line maintained a moderate level of cAMP production. The continual cAMP production provides the basis for the measurement of PDE activity without addition of a TSH receptor agonist. In the absence of a PDE inhibitor, the moderate level of cAMP in this PDE4 cell line is rapidly hydrolyzed by endogenous PDEs, predominantly PDE4 in the host HEK293 [14,21] and is counted as a baseline. In the presence of a PDE4 inhibitor, cAMP accumulates and the increased levels of cAMP bind to and open the CNG cation channels, resulting in an influx of cations including Na^+ and Ca^{2+}. This cation influx triggers a cell membrane depolarization which can be quantified using a fluorescent membrane potential dye that does not require cell wash or medium change steps [22]. Thus, the inhibition of PDE activity can be monitored homogeneously in live cells with a standard fluorescence plate reader.

There are several advantages of this cell-based PDE4 assays compared with other previously reported assays [9,10,23]. First, this cell-based PDE4 assay does not require an addition of exogenous cAMP stimuli such as forskolin (an adenylate cyclase activator) or GPCR agonists because the constitutive activity of co-transfected TSH receptor produces enough

cAMP to generate a measurable signal in the presence of a PDE inhibitor. Second, detection with the membrane potential dye enables homogenous measurement of PDE activity compared to the use of Fura-2 dye or voltage-clamp methods which have lower throughput and require a cell-wash step during the experiments. Third, the assay protocol is substantially simplified and standard fluorescence plate readers can be used, making this assay suitable for miniaturization and high throughput screening.

Cell density optimization

All of our experiments were carried out in the miniaturized 1536-well plate format. The effect of cell density was first tested in the presence or absence of 30 μM (EC_{100}) RO 20-1724, a PDE4 inhibitor. Although the total fluorescence intensity in the presence of 30 μM RO 20-1724 increased with the increase of cell number from 500 to 3000 cells/well, the basal signal also elevated as cell density increased. We found that 1000 cells/well produced a 5.2 fold signal-to-basal ratio which was superior to other cell densities (Fig. 2). Thus, 1000 cells/well was chosen as an optimal cell density in 1536-well plate format for subsequent experiments. We also found that DMSO, the solvent for all of the compounds in our screening collection, had no significant effect on this assay at the concentrations up to 2 %, indicating high tolerance to DMSO for this cell-based PDE4 assay (data not shown).

Time course for PDE4 inhibitors

The time course of 30 μM RO 20-1724 treatment in the PDE4 cells was measured after compound addition every 10 min for 2 hr. We found that after the addition of RO 20-1724, the total fluorescence signal increased rapidly during the first 20 min followed by a slow increase to its peak at 60 min and a gradual decrease afterwards. The basal signal did not change with incubation time (Fig. 3). This result suggested that cAMP pools began to accumulate in the cells after the addition of RO 20-1724 and the assay signal reached equilibrium at approximately 60 min. Other PDE4 inhibitors (Rolipram, Milrinone, Trequinsin, and Etazolate) were examined over a 70 minute timeframe, and the response of all compounds except Trequinsin leveled off after 20 to 30 minutes. Trequinsin showed a gradual increase of activity over 70 minutes with no plateau, however an acceptable signal-to-basal ratio was observed after 20 minutes of exposure (data not shown).

The decrease of the fluorescent signal after 60 min might be caused by the dye leaking out of cells and/or cells becoming less healthy 2 hrs after the membrane potential dye treatment (60 minutes of dye loading and 60 minutes of stimulation). Thus, the response of PDE inhibition is stable and can be measured between 30 and 60 min after compound addition. This provides sufficient time for a measurement with a standard fluorescence plate reader without requiring a kinetic measurement.

TSH and forskolin effects on RO 20-1724 activity

In the cell-based PDE4 assay, a constitutively active GPCR (TSHR) is used to drive the production of cAMP, theoretically making the addition of exogenous GPCR agonists or forskolin unnecessary. However, before using this assay to detect novel PDE inhibitors, we tested it directly by examining whether the activity of a known PDE inhibitor was affected by a GPCR agonist or AC activator. The activity of RO 20-1724 in the presence or absence of 10 nM (EC_{80}) TSH was measured in the PDE4 cell line as well as in the parental cell line stimulated by 200 nM forskolin (EC_{80}). In the PDE4 cell line, the IC_{50} values of RO 20-1724 in the presence and absence of TSH stimulation were 1.72 and 2.39 μM, respectively. The basal response in the TSH stimulated cells was high due to the constitutive TSH receptor activity which reduced the assay window (Fig. 4a). Our results indicated that the potency of RO 20-1724 in the PDE4 cell line remained the same regardless of whether the TSH stimulation was added but that the basal signal was much lower without TSH

stimulation (Fig. 4a). In the parental cell line, RO 20-1724 alone did not produce any measureable response (Fig 4b); indicating that its activity in the PDE4 cell line was dependent on TSH receptor activity. The IC_{50} values of RO 20-1724 in the parental cell line with forskolin stimulation was 1.44 μM (Fig. 4c), similar to that determined from the PDE4 cell line. These results demonstrated that the potency of RO 20-1724 determined in this cell-based PDE4 assay without the stimulation by an exogenous agonist was very similar to the potency determined in the experiments stimulated by the GPCR agonist or forskolin. The lack of requirement for a GPCR agonist or AC activator in this assay has several advantages, including cost and time saving in assay optimization, improved consistency, and less possibility of receptor desensitization, which occurs in many GPCRs including the TSH receptor [24,25] when exposed to high concentrations of agonists.

Compound library screening

A collection of 2187 compounds, most of which had a known pharmacological action, was screened in both the PDE and parental cell lines. All compounds were titrated at 15 concentrations and the screen was performed in the qHTS format [17]. As described in Table 1, 1000 cells/well (3 μl cell suspension) were dispensed into 1536-well assay plates and incubated at 37°C and 5% CO_2 in a cell culture incubator for 24 hrs, followed by addition of 3 μl/well membrane potential dye and incubation at room temperature for 60 min to allow dye equilibration across the cell membrane, as recommended by the membrane potential dye manufacturer. The 15 compound dilutions were then added to the assay plates using a pintool at 23 nl/well, incubated for 60 min at room temperature, and fluorescence intensity was measured in a standard fluorescence plate reader at 530 nm excitation and 590 nm emission. The entire experimental process required reagent additions only, without the need for cell washing or medium aspiration, and was thus easily performed by both a robotic screening system and a semi-automated workstation.

Fig. 5a shows the 1536-well format plate map; columns 1–4 are used for controls, and columns 5–48 for experimental compounds. In a test screening with a DMSO plate, the signal-to-basal ratio was 3.9 fold, CV was 10.5% and Z' factor was 0.50 (Fig. 5b), indicating acceptable performance in 1536-well plate format. These assay statistics (S/B, CV and Z' factor) were very similar in the parental cell line (data not shown). We found that Z' factor was less than 0.5 (0.2 – 0.4) in some plates during the compound library screening. This might be due to the use of HEK293 cells which exhibit reduced attachment on the assay plate after dye treatment and subsequently may have caused an increase in the variation between wells. Use of poly-D-lysine coated plates might be able to improve the cell attachment and thus reduce the variation between wells; however the 1536 well plates would need a customized coating of poly-D-lysine and this was not available at the time of screening. Recently, a similar HEK 293 cell line assayed in non poly-D-lysine coated 1536 well plates was used in a successful qHTS campaign for GPCR agonist screening. The occasional low Z' factor numbers were also found in some of assay plates; however several novel agonists were identified and confirmed from the screen [26].

The screening results from the parental cell line were used to eliminate the nonspecific and false positive compounds including fluorescent compounds, cAMP pathway signal activators (such as endogenous GPCR ligands, forskolin, etc). A total of 35 active compounds with a potency of less than 10 μM and a curve class of 1.1 or 1.2 [17] with maximal inhibition greater than 50% were identified in the PDE4 cell line screen; of these, 17 were also active in the parental cell line. Many of the 17 compounds active in both cell lines have known actions in the cAMP signaling pathway (i.e.; forskolin analogs, isoproterenol, etc) while others are likely fluorescent compounds. Parallel screening of a parental cell line is therefore an effective method for eliminating the false positive compounds in this cell-based PDE4 assay.

Among the 18 remaining compounds, 11 were PDE inhibitors as defined by the compound providers (Table 2). The other 7 actives were reordered and tested in both PDE4 and parental cell lines, as well as in a PDE4 enzyme assay (Table 3). The activities of 4 out of 7 compounds were confirmed in both PDE4 cell line and PDE4 enzyme assay and they were not active in the parental cell line. Among these 4 confirmed compounds, arctigenin was an inhibitor of MKK1 and IkBa phosphorylation and was also previously reported as a putative PDE inhibitor [27]. The other 3 confirmed compounds were all xanthine derivatives that were reported as classical adenosine receptor antagonists as well as inhibiting PDE activity [28–31]. Among the 3 unconfirmed compounds, CB34 was a peripheral benzodiazepine receptor agonist that was also active in both PDE and parental cell lines in the retests although in the primary screen it was not classified as active in the parental cell line due to its borderline activity. The activities of the two other hits were not confirmed in the retests (Table 3). Both of the compounds exhibited borderline activity in the primary screen with weak potency and low efficacy. None of these 3 three compounds (CB34, muscarine chloride, or terfenadine) exhibited PDE inhibitory activity in the purified PDE4 enzyme assay. Hence, 4 out of 7 compounds were confirmed as PDE inhibitors in both the PDE4 cells and the PDE4 enzyme assay, while the activities of other 3 compounds were not confirmed due to the nonspecific action in cells or marginal activity. All primary screen results can be viewed in the PubChem database (AID: 607, http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=607).

Activities of known PDE inhibitors

The selectivity and potency of the PDE inhibitors (as defined by the compound providers) from the cell-based PDE4 assay and counter-screen in the parental cell line are summarized in Table 2. All PDE4 specific inhibitors showed IC_{50} values in the cell-based PDE4 assay within a ten fold window to those reported elsewhere. For example, the IC_{50} values for RO 20-1724, (S)-(−)-Rolipram, Trequinsin, and Etazolate were 1.0, 0.1, 3.2 and 9.9 μM, respectively from this cell-based PDE4 assay, and they showed either no activity or very weak activity in the parental cell line (Fig. 6). Other compounds exhibited higher or lower potencies than reported values, this may be due to differences in the experimental conditions used in different laboratories (such as enzyme subtype, instrumentation, reagents, data normalization, etc). The activities of 5 nonselective PDE inhibitors including Ibudilast, IMBX, 1,3-dipropyl-7-methylxanthine and Pentoxifylline in this assay were also within similar ranges of those reported in the literature (Table 2). The inhibitors specific to other PDE subtypes showed either no activity or very weak activity in this PDE assay. For example, Vinpocetine, a PDE1 inhibitor with 20 μM reported IC_{50}, showed only a 20% response at 33.3 μM in both the PDE and parental cell lines. These results suggest that PDE4 is the predominant PDE enzyme type present in HEK293 cells, consistent with literature reports [14,21].

Conclusion

We have developed and validated a cell-based PDE4 assay in a 1536-well plate format. Cell-based PDE4activity can be determined with a membrane potential dye in a standard fluorescence plate reader; most false positives are easily eliminated by a parallel screen or a counter-screen of the parental cell line. The simplicity of this assay indicates that it is suitable for high throughput screening of large compound libraries in a 1536-well format in order to identify novel cell-permeable PDE4 inhibitors. Preliminary results also suggest that this cell-based PDE4 assay strategy can be extended to other PDEs types, either by heterogonous expression of other PDEs in the TSHR-CNG-HEK293 cell line, or by utilizing this biosensor in cell lines expressing high levels of other PDE family members.

Abbreviations

qHTS	quantitative high throughput screening
SAR	structure-activity relationship
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphates
CNG	cyclic nucleotide gated ion channel
GPCR	G Protein Coupled Receptor
LOPAC	Library of Pharmacologically Active Compounds
PDE	phosphodiesterases

Acknowledgments

We thank Paul Shinn and Adam Yasgar for the compound management and Sam Michael, Carleen Klumpp and Dick Jones for technical assistance with instrumentation. This research was supported by the Molecular Libraries Initiative of the NIH Roadmap for Medical Research and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health.

References

1. Houslay MD, Baillie GS, Maurice DH. cAMP-specific phosphodiesterase-4 enzymes in the cardiovascular system: A molecular toolbox for generating compartmentalized cAMP signaling. Circulation Research. 2007; 100:950–966. [PubMed: 17431197]
2. Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. Pharmacology and Therapeutics. 2006; 109:366–398. [PubMed: 16102838]
3. Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annual review of biochemistry. 2007; 76:481–511.
4. Jeon YH, Heo YS, Kim CM, Hyun YL, Lee TG, Ro S, Cho JM. Phosphodiesterase: Overview of protein structures, potential therapeutic applications and recent progress in drug development. Cellular and Molecular Life Sciences. 2005; 62:1198–1220. [PubMed: 15798894]
5. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use. Pharmacological Reviews. 2006; 58:488–520. [PubMed: 16968949]
6. Menniti FS, Faraci WS, Schmidt CJ. Phosphodiesterases in the CNS: Targets for drug development. Nature Reviews Drug Discovery. 2006; 5:660–670.
7. Johnston PA, Johnston PA. Cellular platforms for HTS: Three case studies. Drug Discovery Today. 2002; 7:353–363. [PubMed: 11893544]
8. Allen RA, Merriman MW, Perry MJ, Owens RJ. Development of a recombinant cell-based system for the characterisation of phosphodiesterase 4 isoforms and evaluation of inhibitors. Biochem Pharmacol. 1999; 57:1375–1382. [PubMed: 10353258]
9. Qiu Y, Bhattacharjee S, Kraft P, John TM, Craig E, Haynes-Johnson D, Guan J, Jiang W, Macielag M, Sui Z, Clancy J, Lundeen S. Characterization of a novel phosphodiesterase type 5 inhibitor: JNJ-10258859. Eur J Pharmacol. 2003; 472:73–80. [PubMed: 12860475]
10. Bora RS, Malik R, Arya R, Gupta D, Singh V, Aggarwal N, Dastidar S, Ray A, Saini KS. A reporter gene assay for screening of PDE4 subtype selective inhibitors. Biochem Biophys Res Commun. 2007; 356:153–158. [PubMed: 17336928]
11. Malik R, Bora RS, Gupta D, Sharma P, Arya R, Chaudhary S, Saini KS. Cloning, stable expression of human phosphodiesterase 7A and development of an assay for screening of PDE7 selective inhibitors. Appl Microbiol Biotechnol. 2007

12. Bora RS, Gupta D, Malik R, Chachra S, Sharma P, Saini KS. Development of a cell-based assay for screening of PDE10 inhibitors using a stable recombinant HEK-293 cell line expressing high level of PDE10A. Biotechnol Appl Biochem. 2007

13. Castro LRV, Verde I, Cooper DMF, Fischmeister R. Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. Circulation. 2006; 113:2221–2228. [PubMed: 16651469]

14. Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW. In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. Journal of General Physiology. 2001; 118:63–77. [PubMed: 11429444]

15. Wunder F, Tersteegen A, Rebmann A, Erb C, Fahrig T, Hendrix M. Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. Molecular Pharmacology. 2005; 68:1775–1781. [PubMed: 16150925]

16. Skoumbourdis AP, Huang R, Southall N, Leister W, Guo V, Cho MH, Inglese J, Nirenberg M, Austin CP, Xia M, Thomas CJ. Identification of a potent new chemotype for the selective inhibition of PDE4. Bioorganic and Medicinal Chemistry Letters. 2008; 18:1297–1303. [PubMed: 18243697]

17. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP. Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103:11473–11478. [PubMed: 16864780]

18. Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. Trends in Biochemical Sciences. 2004; 29:119–126. [PubMed: 15003269]

19. Cetani F, Tonacchera M, Vassart G. Differential effects of NaCl concentration on the constitutive activity of the thyrotropin and the luteinizing hormone/chorionic gonadotropin receptors. FEBS Letters. 1996; 378:27–31. [PubMed: 8549796]

20. Chazenbalk GD, Kakinuma A, Jaume JC, McLachlan SM, Rapoport B. Evidence for negative cooperativity among human thyrotropin receptors overexpressed in mammalian cells. Endocrinology. 1996; 137:4586–4591. [PubMed: 8895321]

21. Rich TC, Karpen JW. High-throughput screening of phosphodiesterase activity in living cells. Methods in molecular biology (Clifton, N.J.). 2005; 307:45–61.

22. Zheng W, Spencer RH, Kiss L. High throughput assay technologies for ion channel drug discovery. Assay and Drug Development Technologies. 2004; 2:543–552. [PubMed: 15671652]

23. Malik R, Bora RS, Gupta D, Sharma P, Arya R, Chaudhary S, Saini KS. Cloning, stable expression of human phosphodiesterase 7A and development of an assay for screening of PDE7 selective inhibitors. Appl Microbiol Biotechnol. 2008; 77:1167–1173. [PubMed: 17952431]

24. Heldin NE, Gustavsson B, Hermansson A, Westermark B. Thyrotropin (TSH)-induced receptor internalization in nonthyroidal cells transfected with a human TSH-receptor complementary deoxyribonucleic acid. Endocrinology. 1994; 134:2032–2036. [PubMed: 8156904]

25. Nagayama Y, Tanaka K, Hara T, Namba H, Yamashita S, Taniyama K, Niwa M. Involvement of G protein-coupled receptor kinase 5 in homologous desensitization of the thyrotropin receptor. Journal of Biological Chemistry. 1996; 271:10143–10148. [PubMed: 8626574]

26. Titus SA, Neumann S, Zheng W, Southall N, Michael S, Klumpp C, Yasgar A, Shinn P, Thomas CJ, Inglese J, Gershengorn MC, Austin CP. Quantitative High Throughput Screening Using a Live Cell cAMP Assay Identifies Small Molecule Agonists of the TSH Receptor. Journal of Biomolecular Screening. 2007

27. Cho JY, Park J, Kim PS, Yoo ES, Baik KU, Park MH. Savinin, a lignan from Pterocarpus santalinus inhibits tumor necrosis factor-? production and T cell proliferation. Biological and Pharmaceutical Bulletin. 2001; 24:167–171. [PubMed: 11217086]

28. Coulson R, Scheinman SJ. Xanthine effects on renal proximal tubular function and cyclic AMP metabolism. Journal of Pharmacology and Experimental Therapeutics. 1989; 248:589–595. [PubMed: 2537403]

29. Howell RE. Multiple mechanisms of xanthine actions on airway reactivity. Journal of Pharmacology and Experimental Therapeutics. 1990; 255:1008–1014. [PubMed: 2175791]

30. Martinson EA, Johnson RA, Wells JN. Potent adenosine receptor antagonists that are selective for the A1 receptor subtype. Molecular Pharmacology. 1987; 31:247–252. [PubMed: 3561384]

31. Ukena D, Schudt C, Sybrecht GW. Adenosine receptor-blocking xanthines as inhibitors of phosphodiesterase isozymes. Biochemical Pharmacology. 1993; 45:847–851. [PubMed: 7680859]

32. Soderling SH, Bayuga SJ, Beavo JA. Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:8991–8996. [PubMed: 9671792]

33. Fryknas M, Rickardson L, Wickstrom M, Dhar S, Lovborg H, Gullbo J, Nygren P, Gustafsson MG, Isaksson A, Larsson R. Phenotype-based screening of mechanistically annotated compounds in combination with gene expression and pathway analysis identifies candidate drug targets in a human squamous carcinoma cell model. J Biomol Screen. 2006; 11:457–468. [PubMed: 16928983]

34. Mochizuki N, Uchida S, Miyata H. Cardiovascular effects of NSP-804 and NSP-805, novel cardiotonic agents with vasodilator properties. Journal of Cardiovascular Pharmacology. 1993; 21:983–995. [PubMed: 7687727]

35. Holck M, Thorens S, Muggli R, Eigenmann R. Studies on the mechanism of positive inotropic activity of Ro 13-6438, a structurally novel cardiotonic agent with vasodilating properties. Journal of Cardiovascular Pharmacology. 1984; 6:520–530. [PubMed: 6202981]

36. Souness JE, Houghton C, Sardar N, Withnall MT. Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer. British Journal of Pharmacology. 1997; 121:743–750. [PubMed: 9208143]

37. Meskini N, Nemoz G, Okyayuz-Baklouti I, Lagarde M, Prigent AF. Phosphodiesterase inhibitory profile of some related xanthine derivatives pharmacologically active on the peripheral microcirculation. Biochem Pharmacol. 1994; 47:781–788. [PubMed: 8135854]

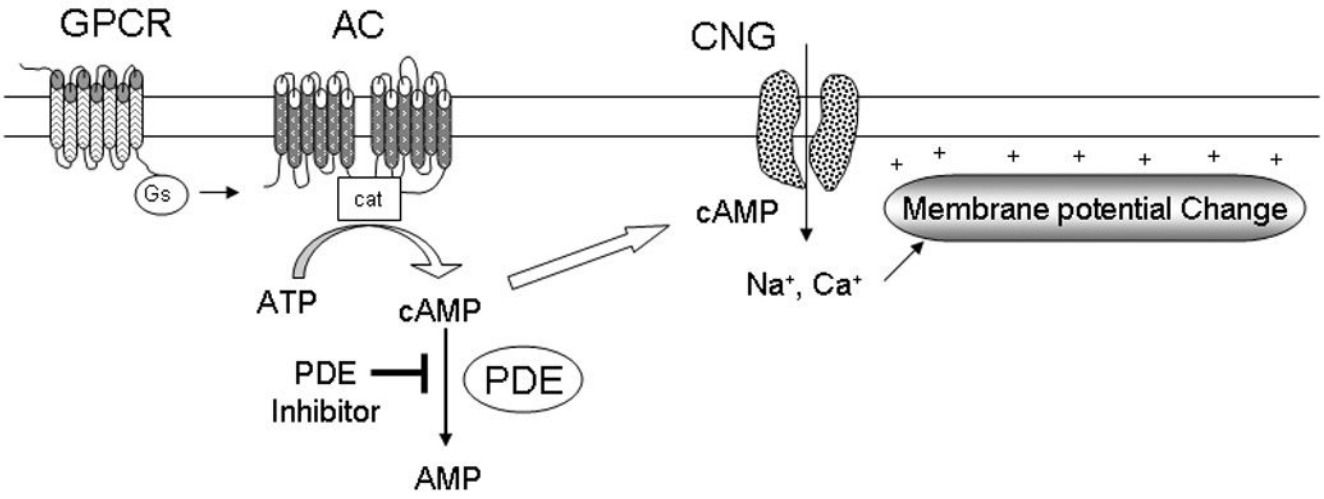

Fig. 1.

Schematic diagram of the detection mechanism of the cell-based PDE4 assay. The thyroid stimulating hormone (TSH) receptor was co-transfected in HEK293 cells with a modified CNG channel which serves as a biosensor of intracellular cAMP levels. This transfected TSH receptor has a low level of constitutive activity that stimulates adenylate cyclase (AC) to produce a small amount of cAMP. In the absence of a PDE inhibitor, this small amount of cAMP is quickly hydrolyzed by PDE, preventing activation of the CNG cation channel. In the presence of a PDE inhibitor, the cAMP level rises, activating the CNG cation channel and resulting in membrane depolarization. A membrane potential dye is used to monitor the change in the membrane potential that is proportional to the inhibition of PDE by the PDE inhibitor.

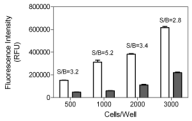

Fig. 2.

The effects of cell density on the cell-based PDE4 assay. Cells were plated at 500, 1000, 2000 and 3000 cells/well in a 1536 well plate and incubated for 24 hours before the addition of membrane potential dye and a PDE4 inhibitor. The open bar represents the total fluorescence signal in the presence of 30 μM RO 20-1724 and the closed bar is the basal fluorescence signal in the absence of RO 20-1724. The signal to background ratio (S/B) was calculated by dividing the average of stimulation (4 replicates) by the average of the untreated controls (4 replicates). RFU was the relative fluorescence unit measured at 590 nm emission.

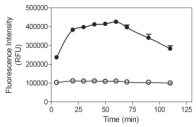

Fig. 3.

Time course of the PDE inhibitor incubation. Cells were seeded at 1000 cells/well in 1536 well plates. After 24-hour incubation, the membrane potential dye and 30 μM RO 20-1724 were added. The fluorescence signals of the membrane potential dye were measured every 10 minutes from 10 to 120 minutes after the addition of RO 20-1724 (●) or DMSO as a negative control (○).

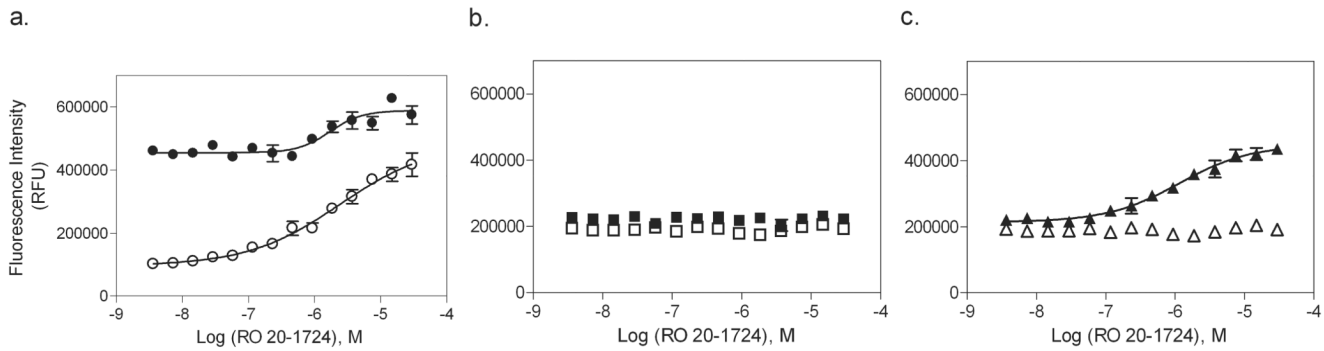

Fig. 4.

Concentration responses of RO 20-1724 determined in the PDE4 cell line (TSHR-CNG-HEK293) and parental cell line (CNG-HEK293). Cells were plated at 1000 cells/well in 1536-well plates and incubated for 24 hours. (**a**) RO 20-1724 responses to TSH in the PDE4 cell line. The IC_{50} values of RO 20-1724 were 1.72 and 2.39 μM in the presence (●) or absence (○) of 30 nM (IC_{80}) TSH stimulation, respectively. (**b**) RO 20-1724 responses to TSH in the parental cell line. Parental cells did not respond to TSH stimulation (with (■) or without (□) 30 nM TSH), respectively. (**c**) RO 20-1724 responses to forskolin in the parental cell line. The IC_{50} value of RO 20-1724 was 1.44 μM (▲) in the presence of 0.2 μM forskolin. In the absence of forskolin, RO 20-1724 did not produce any signal in this cell line (△).

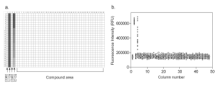

Fig. 5.

Plate map and the results of a DMSO plate screen in 1536-well plate format. (**a**) In a 1536 well plate, 30 μM RO 20-1724 (EC_{100}) were added to all the wells in column 2 as the positive control (RO (1)). The wells in Column 1 and 3 received DMSO solution as the negative control. The wells in column 4 received a titration of RO 20-1724 (1:2 dilution × 16 concentrations and n=2) as a control concentration-response curve (RO (2)). 1408 wells in column 5 through column 48 could be added with the compounds in DMSO solution (DMSO only for this test experiment). (**b**) Scatter plot of a DMSO plate screen in the PDE4 cell-based assay. The signal to background ratio was 3.9 fold and the Z' factor was 0.46.

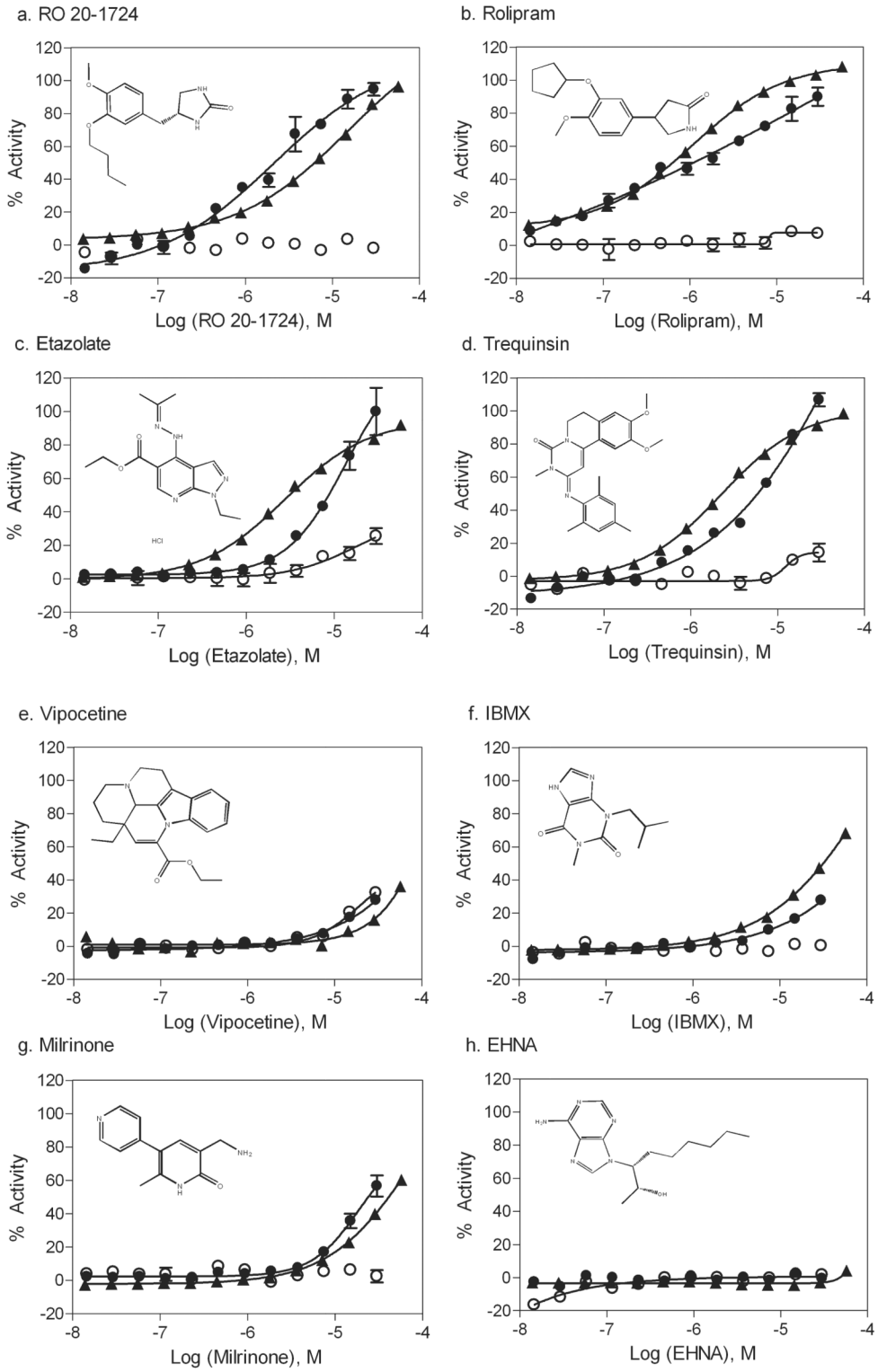

Fig. 6.

Concentration response curves of eight representative known PDE4 inhibitors determined from the PDE4 cell-based assay. The experiments were carried out in the PDE4 cell line (●)

or purified PDE4 A1A enzyme (▲) for the measurement of PDE inhibition or in the parental cell line (○) as a control. (a) RO 20-1724. (b) Rolipram, a PDE4 inhibitor. (c) Etazolate, a PDE4 inhibitor (d) Trequinsin, a PDE3,4 inhibitor. (e) Vinpocetine, a PDE1 inhibitor. (f) IMBX, a nonselective PDE inhibitor. (g) Milrinone, a PDE3 inhibitor. (h) EHNA, a PDE2 inhibitor. Data were calculated from 4 samples per point.

Table 1

The cell-based PDE4 assay protocol in 1536-well plate format.

Step	Parameter	Value	Description
1	Reagent	3 μl	1000 cells/well
2	Incubation	24 hours	37°C 5% CO_2
3	Reagent	3 μl	Membrane Potential Dye
4	Incubation	60 min	Room temperature
5	Compound	23 nl	Column 1 to 4 controls and column 5–48 compounds
6	Incubation	60 min	Room temperature
7	Detector	Ex=535 nm Em=590 nm	Envision plate reader

Table 2

Activities of known PDE inhibitors determined from qHTS.

Compound #	Compound Name	Specificity (PDE isotype)	IC_{50} (μM) in PDE4 cells	% Max. Resp. in PDE4 cells	IC_{50} (μM) in parental cells	% Max. Resp. in parental cells	IC_{50} (μM, reported)	Ref.
1.	(S)-(−)-Rolipram (T-0905)	4	0.1	134	Inact		2.0	[32]
2.	(R)-(−)-Rolipram (T-1349)	4	0.02	121	Inact		0.08	(Tocris)
3.	Rolipram (LOPAC R-6250)	4	0.3	98	Inact		0.8	[32]
4.	(S)-(+)-Rolipram (T-1350)	4	0.3	116	Inact		3.0	(Tocris)
5.	Ro 20-1724	4	1.0	115	Inact		1.9	[32]
6.	YM 976	4	0.01	92	Inact		2 nM	[32]
7.	ICI 63197	4	0.2	68	Inact		35 nM	(Tocris)
8.	Etazolate hydrochloride	4	9.9	105	Inact	18	2.0	(Tocris)
9.	Trequinsin hydrochloride	3, (4)	3.2	140	15.8	14	3 nM, 1 μM	(Tocris)
10.	Zardaverine	3, (4)	1.0	128	Inact		0.5, (0.8)	(Tocris)
11.	Vinpocetine	1	Inact	21	Inact		20	(Tocris)
12.	MMPX	1	Inact.		Inact		5.2	(Tocris)
13.	8-Methoxymethyl IBMX	1	Inact		Inact		4.0	(Calbiochem)
14.	Calmidazolium chloride	1	Inact		Inact		10 nM	(Calbiochem)
15.	EHNA	2	Inact		Inact		0.8	(Calbiochem)
16.	Imazodan	2	Inact		Inact		6.5	[33]
17.	Cilostazol	3	11.9	59	20	22	0.2	(Tocris)
18.	Milrinone	3	6.3	52	Inact		0.3	(Tocris), [34]
19.	Cilostamide	3	Inact		Inact		0.1	(Tocris)
20.	Siguazodan	3	Inact		Inact		0.1	(Tocris)
21.	Enoximone	3	Inact		Inact		1.0	[32]
22.	Quazinone	3	Inact		Inact		0.6	[35]
23.	T-1032	5	10	82	Inact		1 nM	[36]
24.	T 0156 hydrochloride	5	Inact		Inact		0.2	(Tocris)
25.	MY-5445	5	Inact		Inact		0.5	(Tocris)

Compound #	Compound Name	Specificity (PDE isotype)	IC_{50} (μM) in PDE4 cells	% Max. Resp. in PDE4 cells	IC_{50} (μM) in parental cells	% Max. Resp. in parental cells	IC_{50} (μM, reported)	Ref.
26.	T-0156	5	Inact		Inact		0.2	(Tocris)
27.	Zaprinast	6,5,11,9	Inact		Inact		0.2, 0.8, 12, 29	(Tocris)
28.	Papaverine hydrochloride	Nonselect	15.8	155	Inact		0.4 – 20	[32]
29.	Ibudilast	Nonselect	2.5	135	Inact		12.0	(Tocris)
30.	IMBX	Nonselect	12.5	119	Inact		25.0	(Calbiochem)
31.	1,3-Diethyl-8-phenylxanthine	Nonselect	2.0	113	Inact		10.0	[31]
32.	Pentoxifylline	Nonselect	Inact		Inact		39.0	[37]

Note : Compound numbers in **bold** represent the 11 PDE inhibitors that passed screening cutoff criteria for data analysis. % Max. Resp. - % of maximal response normalized to the maximal response of 30 μM RO 20-1724. *Nonselect* – nonselective PDE inhibitor. *Inact* – inactive in the assay. (Tocris) Tocris website; www.tocris.com. Details in the Signal Transduction section. (Calbiochem) Calbiochem website; www.merckbiosciences.co.uk/html/cbc/other_inhibitors_phosphodiesterase.htm.

Table 3

Activities of 7 compounds determined in the follow-up assays.

Compound #	Compound Name	Compound Activity Description	IC_{50} (μM) PDE4 cells	Max % Resp. PDE4 cells	IC_{50} (μM) Parent cells	Max % Resp. Parent cells	IC_{50} (μM) PDE enzyme	Max % Resp. PDE enzyme
1	Arctigenin	Inhibitor of IkBa phosphorylation. Also inhibits MKK1	76.4	53	Inact.		8.8	94
2	DPCPX	Selective A1 adenosine receptor antagonist	8.1	52	Inact.		2.1	41
3	DPX	A1 Adenosine receptor antagonist	6.2	66	Inact.		0.4	93
4	FSCPX	Irreversible A1 adenosine receptor antagonist	1.1	58	Inact.		0.9	33
5	CB 34	Selective high affinity ligand at peripheral benzodiazepine receptors.	8.6	140	10.5	127	Inact.	
6	(+/−)-Muscarine chloride	Muscarinic acetylcholine receptor agonist	17.8	53	12.2	29	Inact.	
7	Terfenadine	Non-sedating H1 histamine receptor antagonist	4.1	20	8.4	35	Inact.	

Notes: Compounds tested in PDE, parental cell line, and enzyme assay were described in Materials and Methods. Compound activity description was obtained from the compound provider. DPX: 1,3-Diethyl-8-phenylxanthine, DPCPX: 8-Cyclopentyl-1,3-dipropylxanthine, FSCPX: 8-Cyclopentyl-N^3-[3-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine. CB 34: N,N-Dipropyl-2-(4-chlorophenyl)-6,8-dichloro-imidazo[1,2-a]pyridine-3-acetamide. % Max. Resp. - % of maximal response normalized to the maximal response of 30 μM RO 20-1724 (PDE4 cells and enzyme) or 10 μM forskolin (parental cells).