Identification of biologically-active PDE11-selective inhibitors using a yeast-based high throughput screen

Author: Ozge Ceyhan

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IDENTIFICATION OF BIOLOGICALLY-ACTIVE PDE11-SELECTIVE INHIBITORS
USING A YEAST-BASED HIGH THROUGHPUT SCREEN

a dissertation

by

OZGE CEYHAN

submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

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ABSTRACT

Identification of biologically-active PDE11-selective inhibitors using a yeast-based high throughput screen

By Ozge Ceyhan

Thesis advisor: Charles S. Hoffman, Ph.D.

The biological roles of the most recently discovered mammalian cyclic nucleotide phosphodiesterase (PDE) family, PDE11, are poorly understood, in part due to the lack of selective inhibitors. To address this need for such compounds I completed a ~200,000 compound high throughput screen (HTS) for PDE11 inhibitors using a yeast-based growth assay. Further characterization of lead candidates using both growth-based assays in the fission yeast Schizosaccharomyces pombe and in vitro enzyme assays identified four potent and selective PDE11 inhibitors. I examined the effect of these compounds on human adrenocortical cells, where PDE11 is believed to regulate cortisol levels. One compound, along with two structural analogs, elevates cAMP levels and cortisol production through PDE11 inhibition, thus phenocopying the behavior of adrenocortical tumors associated with Cushing syndrome. These compounds can be used as research tools to study the biological function of PDE11, and can also serve as leads to develop therapeutic compounds for the treatment of adrenal insufficiencies. This study further validates the yeast-based HTS platform as a powerful tool for the discovery of potent, selective and biologically-active PDE inhibitors.
To my parents and my brother, who have given me endless support and encouragement to achieve my goals...
ACKNOWLEDGEMENT

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of view” in my committee meetings. I am indebted to Dr. Adam Lerner, who has been very kind to join my committee from Boston University. With his expertise in PDE field and unique perspective as a physician, he has been extremely helpful in guiding my project in the right direction. I am very grateful to all my committee members for their kindness and assistance in organizing my meetings and thesis defense.

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CHAPTER ONE

INTRODUCTION

1.1. CYCLIC NUCLEOTIDE SIGNALING IN HUMANS

Cyclic AMP (cAMP) and cyclic GMP (cGMP) are important second messengers that regulate a myriad of processes and cellular functions in humans, such as cell proliferation, differentiation, apoptosis, muscle contraction, cognition, memory, inflammation, metabolism, and endocrine signaling (Beavo et al., 2007; Biel, 2009; Gomperts et al., 2002; Hofmann, 2005; Lucas et al., 2000; Steinberg and Kemp, 2009).

Since the discovery of cAMP in 1958 by Sutherland and Rall (Sutherland and Rall, 1958), which was followed by the identification of cGMP in 1963 by Ashman et al. (Ashman et al., 1963), numerous studies have established their roles in mediating intracellular responses to different kinds of extracellular signals in a wide range of physiological events.

In eukaryotes, most cAMP signaling cascades begin with the binding of a ligand, such as a hormone or a neurotransmitter, to a G-protein coupled receptor (Figure 1A), leading to activation of adenylate cyclases by the Gα subunit of a heterotrimeric G-protein, which produces cAMP from ATP. Intracellular targets of cAMP include cyclic nucleotide-gated ion channels (cN-gated ion channels), exchange proteins activated by cAMP (EPACs), and Protein Kinase A (PKA) (Gomperts et al., 2002). Activation of PKA occurs
by binding of cAMP to the regulatory subunit of the enzyme, which causes its
dissociation from the catalytic subunit that will phosphorylate target proteins (Carling
et al., 2011; Steinberg and Kemp, 2009). Similarly, cGMP is produced by guanylate
cyclases upon activation by ligands such as nitric oxide, calcium ions, and natriuretic
and intestinal peptides, and regulates the activity of cN-gated ion channels and Protein
Kinase G (PKG) (Schmidt et al., 2009) (Figure 1B).

cAMP and cGMP translate extracellular signals into intracellular responses via their
activities on several effector proteins. Activation of cN-gated ion channels by these
molecules is directly coupled to the influx of extracellular cations into the cytoplasm
and depolarization of the plasma membrane (Biel, 2009). The hyperpolarization-
activated channels, which are found in neurons and cardiac cells, are activated by
cAMP-binding and open at hyperpolarized membrane potentials. The cN-gated ion
channels in retinal photoreceptors and olfactory neurons have a preference for cGMP-
binding, and play a key role in visual and olfactory signal transduction by controlling
the membrane potential and calcium concentrations of odor and photoreceptors (Lucas
et al., 2000). In addition to its central role in the visual and olfactory system, cGMP
controls a myriad of events in nitric oxide- and natriuretic peptide-signaling pathways
through activation of PKG, such as smooth muscle tone, platelet aggregation, renin
secretion, intestinal fluid and electrolyte homeostasis, bone growth, and circadian clock
(Hofmann, 2005; Schmidt et al., 2009). On the other hand, PKA is responsible for
mediating the majority of the signals conveyed by cAMP, via regulating the activity of
various transcription factors, cell cycle control proteins and metabolic enzymes (Carling et al., 2011). PKA acts as a sensor for the energy/nutrient needs of the cells and various extracellular stimuli, by getting activated in response to stresses such as hypoxia, muscle contraction, or glucose deprivation, and signals like hormones and cytokines (Asher and Schibler, 2011; Hardie, 2011). It regulates various pathways that control cell cycle, growth, apoptosis, hormone secretion, and metabolism by direct phosphorylation of rate-limiting enzymes as well as at the transcriptional level by acting on transcription factors, such as the cAMP response element-binding (CREB) family of proteins (Canto and Auwerx, 2010; Steinberg and Kemp, 2009). Another group of effector proteins activated by cAMP are EPACs, which are specific guanine nucleotide exchange factors for the Ras GTPase homologues Rap1 and Rap2. Upon activation by EPACs, these proteins control downstream signaling pathways that regulate cell growth, division, differentiation, and adhesion (Borland et al., 2009). By controlling the activity of these important effector proteins in different tissues, cAMP and cGMP play major roles in key biological processes both at cellular and organismic levels.
**Figure 1: Schematic representation of cyclic nucleotide signaling.** (A) cAMP signaling is initiated by the binding of an extracellular ligand to a G-protein coupled receptor in the cell membrane. This causes activation of the G-protein by binding of GTP, which activates adenylate cyclases to produce cAMP molecules. cAMP controls various cellular processes through the activity of cN-gated ion channels, EPACs, and PKA. Intracellular levels of these second messengers are regulated by the balance between their synthesis by adenylate cyclases and degradation by phosphodiesterases. (B) cGMP is produced by guanylate cyclases in response to ligands such as nitric oxide. cGMP activates cN-gated ion channels and PKG, which control processes such as smooth muscle tone and electrolyte homeostasis. cGMP levels are also regulated via their hydrolysis by specific phosphodiesterases.
Figure 1

A

First messenger

G-protein coupled receptor

Activated G-protein

Adenylate cyclase

ATP

cAMP

PDE

5’AMP

cN-gated ion channels

Epac

Protein Kinase A

Transcription

Metabolism

Cellular architecture

Apoptosis

Cell cycle

B

Nitric Oxide

Guanylate cyclase

GTP

cGMP

PDE

5’GMP

cN-gated ion channels

Protein Kinase G

Circadian clock

Muscle tone

Fluid/electrolyte homeostasis
1.2. MAMMALIAN CYCLIC NUCLEOTIDE PHOSPHODIESTERASES (PDES):

PROPERTIES AND THERAPEUTIC VALUE

The intracellular levels of cAMP and cGMP are determined by the balance between their synthesis by adenylate or guanylate cyclases and their degradation by PDEs. PDEs are the enzymes that catalyze the hydrolysis of the 3’ cyclic phosphate bonds of cAMP and cGMP molecules and convert them into 3’, 5’ cyclic monophosphates (Figure 2). In mammals, 21 genes encode ~100 PDE isoforms, that are grouped into 11 families based on their substrate-specificity, sequence conservation, and regulatory properties (Table 1) (Beavo et al., 2007; Bender and Beavo, 2006; Francis et al., 2011; Lerner and Epstein, 2006). Based on their preference for cAMP or cGMP as substrates, PDEs can be defined as either cAMP-specific (PDEs 4, 7, and 8), cGMP-specific (PDEs 5, 6, and 9), or dual-specific (PDEs 1, 2, 3, 10, and 11). Besides their control at the genetic level, PDEs are also regulated by various post-translational modifications, protein-protein interactions, and allosteric binding of cyclic nucleotides or Ca^{2+}/calmodulin.

The intracellular and subcellular localization of PDEs is key to their physiological functions. There are various different PDE isoforms in a cell, including both cAMP- and cGMP-hydrolyzing enzymes. Specific PDEs are localized to distinct compartments of cells, where they control the amplitude and temporal duration of cyclic nucleotide pools (Houslay, 2010). This intracellular localization pattern creates microenvironments for a variety of signaling cascades and allows different PDEs to mediate particular processes within a cell (Francis et al., 2011; Lerner and Epstein, 2006).
Figure 2: PDEs hydrolyze the 3’ cyclic phosphate bond of cAMP and cGMP.

Structures of (A) cAMP and (B) cGMP. The arrow indicates the 3’ cyclic phosphate bonds of cAMP and cGMP molecules that are hydrolyzed by PDE enzymes, converting them into 5’AMP and 5’GMP, respectively.
Figure 2

A

\[ \text{cAMP} \]

B

\[ \text{cGMP} \]
Table 1: Kinetic and regulatory properties of the PDE superfamily (Bender and Beavo, 2006; Francis et al., 2011; Lerner and Epstein, 2006).

<table>
<thead>
<tr>
<th>PDE family</th>
<th>Isoforms</th>
<th>Substrate specificity</th>
<th>Km (µM)</th>
<th>Regulatory properties</th>
<th>Tissue distribution</th>
<th>Selective Inhibitors</th>
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<tr>
<td>PDE1</td>
<td>PDE1A-C</td>
<td>cAMP=cGMP</td>
<td>1-100</td>
<td>1-5</td>
<td>Smooth muscle, brain, heart</td>
<td>Vinpocetine, IC224</td>
</tr>
<tr>
<td>PDE2</td>
<td>PDE2A</td>
<td>cAMP=cGMP</td>
<td>30</td>
<td>10</td>
<td>Brain, heart, macrophage subsets</td>
<td>EHNA, BAY60-7550</td>
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<tr>
<td>PDE3</td>
<td>PDE3A/B</td>
<td>cAMP=cGMP</td>
<td>0.2-0.4</td>
<td>0.02-0.2</td>
<td>Vascular smooth muscle, heart, adipocytes, kidney</td>
<td>Cilostamide, Milrinone, Trequinsin</td>
</tr>
<tr>
<td>PDE4</td>
<td>PDE4A-D</td>
<td>cAMP&gt;cGMP</td>
<td>1-5</td>
<td>-</td>
<td>Widely expressed in various tissues, immune cells, brain, lung, testis</td>
<td>Rolipram, Roflumilast, Cilomilast</td>
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<tr>
<td>PDE5</td>
<td>PDE5A</td>
<td>cGMP&gt;cAMP</td>
<td>-</td>
<td>5</td>
<td>Vascular smooth muscle, platelets, heart, skeletal muscle</td>
<td>Zaprinast, Sildenafil, Verdanafil, Tadalafil</td>
</tr>
<tr>
<td>Gene</td>
<td>Type</td>
<td>Catabolic Action</td>
<td>Km</td>
<td>Localization</td>
<td>Function</td>
<td>Inhibition</td>
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<td>PDE6</td>
<td>PDE6A-C</td>
<td>cGMP&gt;cAMP</td>
<td>-</td>
<td>15-17</td>
<td>Rod/cone cells of the retina, pineal gland</td>
<td>PDE6 inhibition is undesirable due to its key role in the visual system</td>
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<td>PDE7A/B</td>
<td>cAMP&gt;cGMP</td>
<td>0.03-0.2</td>
<td></td>
<td>Immune cells, heart, skeletal muscle</td>
<td>BRL 50481</td>
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<td>PDE8</td>
<td>PDE8A/B</td>
<td>cAMP&gt;cGMP</td>
<td>0.06-0.1</td>
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<td>Brain, thyroid</td>
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<td>PDE9</td>
<td>PDE9A</td>
<td>cGMP&gt;cAMP</td>
<td>-</td>
<td>0.07</td>
<td>Kidney, brain, spleen, prostate</td>
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<td>PDE10</td>
<td>PDE10</td>
<td>cAMP=cGMP</td>
<td>0.26-1.1</td>
<td>14</td>
<td>Brain, testis, heart, thyroid</td>
<td>PF-2545920 MP-10</td>
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<tr>
<td>PDE11</td>
<td>PDE11A</td>
<td>cAMP=cGMP</td>
<td>0.4-3</td>
<td>0.3-2</td>
<td>Skeletal muscle, prostate, testis, liver, pituitary and adrenal glands</td>
<td>None</td>
</tr>
</tbody>
</table>
PDEs have been recognized as valuable targets for the treatment of a wide range of conditions including inflammatory diseases such as chronic obstructive pulmonary disease (COPD), asthma, pulmonary hypertension; neurological diseases such as depression, memory loss, and schizophrenia; metabolic diseases such as diabetes; erectile dysfunction, and various forms of cancer (Beavo et al., 2007; Bender and Beavo, 2006; Lerner and Epstein, 2006; O'Donnell and Zhang, 2004; Page and Spina, 2011; Rose et al., 2005; Spina, 2008). There are several important reasons that make PDEs good therapeutic targets. Firstly, cAMP and cGMP regulate various key biological processes, and the most rapid and efficient way to alter the intracellular concentration of these second messengers is to target their rate of degradation by PDE enzymes. In addition, the diversity and the unique tissue expression and subcellular localization patterns of PDEs allow individual isoforms to regulate distinct physiological functions, and link them to different pathological conditions (Bender and Beavo, 2006). Therefore, selective PDE inhibitors have the potential to provide therapeutic benefit without affecting other physiological processes and causing side effects. PDE5-specific inhibitors have long been used for the treatment of erectile dysfunction (Bender and Beavo, 2006; Bischoff, 2004), and one PDE4-specific inhibitor has recently been FDA-approved for the treatment of COPD (Diamant and Spina, 2011).

Understanding the functional role of different PDE isoenzymes has been complicated by the diversity of the PDE superfamily and the presence of multiple isoforms within a single cell. In addition to their therapeutic potential, inhibitors that are selective for
individual PDE families are also beneficial research tools for elucidating the physiological role of particular PDEs. Among the 11 PDE families, PDE11 is the only family that currently lacks specific inhibitors. None of the commonly used nonselective inhibitors are very effective on PDE11, and no PDE11-selective inhibitors are available. By causing an acute loss of function, selective PDE inhibitors provide unique opportunities to demonstrate the functional role of an isoenzyme, which has been difficult using knock-out animal models due to compensation by other PDEs during development.

1.3. PHOSPHODIESTERASE 11 (PDE11) FAMILY

1.3.1. Variants and structure

PDE11 is the most recently discovered phosphodiesterase family (Fawcett et al., 2000). Four human PDE11 variants have been identified that are produced from a single gene via alternative splicing (PDE11A1-4) (Weeks et al., 2007) (Figure 3). PDE11A isoforms have a conserved carboxyl terminus, but differ in their amino terminal regions (Fawcett et al., 2000; Hetman et al., 2000; Yuasa et al., 2000). The longest isoform, PDE11A4, consists of 934 amino acids. The other three variants, PDE11A1, PDE11A2, and PDE11A3, are truncations of the long isoform with varying lengths of N-terminal regions, and consist of 490, 576, and 684 amino acids, respectively.

PDE11A4 has a C-terminal catalytic domain and an N-terminal regulatory region that contains two consensus phosphorylation sites for Protein Kinase A (PKA) or Protein
Kinase G (PKG), and two GAF (cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclase, and *Escherichia coli* FhlA) domains (GAF-A and GAF-B) (Fawcett et al., 2000; Hetman et al., 2000; Yuasa et al., 2000). The other three variants contain either intact or portions of a GAF sequence at the N-terminus. The majority of GAF domains in mammalian proteins are found in phosphodiesterases (Zoraghi et al., 2004). Five PDE families (PDEs 2, 5, 6, 10, and 11) contain tandem GAF domain sequences. In PDEs 2, 5, and 6, the GAF domains contain a non-catalytic cGMP-binding site. The binding of cGMP to the GAF domains in PDE2A and PDE5A stimulates the catalytic activity of the enzymes, while it triggers the binding of the inhibitory domain in PDE6 (Zoraghi et al., 2004). The GAF domains in PDE10A and PDE11A bind cAMP and cGMP respectively with high affinity, however ligand binding does not stimulate catalytic activity in these enzyme families (Matthiesen and Nielsen, 2009). The functional significance of the phosphorylation sites and the GAF domains on PDE11 isoforms remain unknown.
Figure 3: Schematic representation of PDE11A variants. Black shaded region represents the conserved catalytic domain of PDE11A enzymes. S117 and S162 are the two consensus phosphorylation sites for PKA and PKG. PDE11A4 is the only isoform that has two intact GAF (cGMP-binding phosphodiesterase, Anabaena adenylyl cyclase, and Escherichia coli FhlA) domains; and contains all residues present in PDE11A1, and PDE11A2. PDE11A3 contains a unique 54 aminoacid insert at the N-terminus.
Figure 3

[Diagram showing the structure of PDE11A4, PDE11A3, PDE11A2, and PDE11A1 with N-term domain and Catalytic domain labels.]

- PDE11A4
  - N-term domain
  - Catalytic domain
  - S117, S162

- PDE11A3
  - N-term domain
  - Catalytic domain
  - GAF B

- PDE11A2
  - N-term domain
  - Catalytic domain
  - GAF B

- PDE11A1
  - N-term domain
  - Catalytic domain
  - GAF B
1.3.2. Sequence similarity with other PDEs

Sequence alignments of human PDE11A proteins reveal that this family is most similar to PDE5, then to PDE6 and PDE10 enzymes (Table 2), especially across the catalytic domain, the most highly conserved region among PDE families. The catalytic domain of PDE11A is 50% identical to that of PDE5A, 41-44% identical to PDE6 isoforms, and 41% identical to PDE10A.

1.3.3. Catalytic properties

PDE11 enzymes hydrolyze both cAMP and cGMP (Fawcett et al., 2000; Yuasa et al., 2000). The $K_m$ values for the two substrates range between 0.4-3 $\mu$M for cAMP and between 0.3-2 $\mu$M for cGMP hydrolysis, depending on the variant and tissue studied. Although PDE11A variants display small variations in their catalytic activities, their $K_m$ and relative cGMP/cAMP $V_{max}$ values are nearly equal (Weeks et al., 2007) (Table 3). PDE11 enzymes have relatively low $V_{max}$ values compared to the rest of the PDE superfamily, as even the most active variant, PDE11A4, has a $V_{max}$ of 270 nmol/min/mg (Bender and Beavo, 2006; Yuasa et al., 2000).

Recombinant PDE11 enzymes have been characterized for their inhibition profile by well-known nonselective PDE inhibitors (D’Andrea et al., 2005; Hetman et al., 2000; Yuasa et al., 2000). PDE11 is insensitive (IC$_{50}$$>$100 $\mu$M) to Vinpocetin, EHNA, Milrinone, and Rolipram, which are selective inhibitors for the PDE1, 2, 3, and 4 families.
respectively; moderately sensitive to the nonselective PDE inhibitor IBMX (IC\textsubscript{50} ~25-50 µM) and PDE5/6 inhibitor Zaprinast (IC\textsubscript{50} ~2-10 µM); and highly sensitive to the PDE5/6/9/10 inhibitor Dipyridamole (IC\textsubscript{50} ~0.3-1 µM).

The potency of the PDE5-specific inhibitors that are currently in use for the treatment of erectile dysfunction on PDE11 inhibition has raised concerns about their clinical activities and potential side effects. Among these inhibitors, Sildenafil and Verdanafil have PDE11/PDE5 IC\textsubscript{50} ratios of 203 and 346, respectively. However, Tadalafil inhibits PDE11 with an IC\textsubscript{50} of 37 nM, and its selectivity ratio for PDE5 with respect to PDE11 is only 5 (Bischoff, 2004). Some of the side effects by Tadalafil have been attributed to its significant activity against PDE11, however this issue has not been clarified to date, in part due to the lack of PDE11-selective inhibitors that can distinguish PDE11-dependent responses.

1.3.4. **Biological roles of PDE11 in different tissues and diseases**

PDE11A expression has been identified both at mRNA and protein levels in several tissues (D’Andrea et al., 2005; Fawcett et al., 2000; Lakics et al., 2010). These studies have demonstrated that PDE11 is expressed in skeletal muscle, prostate, testis, brain, kidney, liver, pancreas, lymphoid cells, pituitary and adrenal glands. The lack of selective PDE11 inhibitors has impeded functional studies of this enzyme, therefore the biological roles of PDE11 in these tissues are still poorly understood. However, a number of recent genome-wide association studies (GWASs) that link PDE11 defects
with different phenotypes, as well as studies using PDE11 knockout mice have provided insights into the functional significance of the enzyme in various physiological processes and pathologies.

1.3.4.1. The role of PDE11 in sperm physiology

A physiological role for PDE11 in murine sperm physiology has been demonstrated by Wayman et al., by generation of a PDE11 knockout mouse (Wayman et al., 2005). They have documented high levels of PDE11 expression in the testis, prostate, and developing spermatozoa of wild-type mice, and modest changes in ejaculated sperm from the PDE11 knockout mice, including a reduction in forward motility (30%) and concentration of live sperm (20%), as well as increased spontaneous capacitance (29% versus 19% in wild-type mice). Despite these alterations in sperm physiology, PDE11-null mice were generally normal, and their fertility rates were comparable to those of wild-type mice; although the absence of more dramatic phenotypes may be due to compensation by other PDEs during development that can replace functions normally provided by PDE11.

1.3.4.2. PDE11A genetic alterations as risk factors for prostatic cancers and testicular germ cell tumors

The PDE11A gene is one of 30 genes that are highly linked to prostatic cancer in genome-wide association studies (Eeles et al., 2009; Faucz et al., 2011), and its transcripts are in the top 1% of mRNAs that are down-regulated in this disease.
compared to normal prostate, according to expression meta-analyses (Gorlov et al., 2009). In addition, \textit{PDE11A}-inactivating variants have been observed at significantly higher frequencies among patients with prostate cancer and familial and bilateral testicular germ cell tumors (TGCT) (Faucz et al., 2011; Horvath et al., 2009). Each of the missense mutations in these variants resulted in lower levels of PDE11 expression or activity. These studies suggest that \textit{PDE11A}-inactivating genetic alterations may modify the risks for prostate cancer and TGCT.

\textbf{1.3.4.3. PDE11A in the brain}

Phosphodiesterase signaling has been shown to be altered in the cerebella of subjects with schizophrenia, bipolar disorder, and major depression (Fatemi et al., 2010). Using qRT-PCR analysis, Fatemi et al. demonstrated that PDE11A was upregulated in the cerebella from subjects with bipolar disorder and schizophrenia. The involvement of PDEs in susceptibility to psychiatric diseases have also been demonstrated by GWASs. Results by Wong \textit{et al.} suggest that \textit{PDE11A} sequence variants may be associated with major depression and response to antidepressant treatment response (Wong et al., 2006).

Kelly \textit{et al.} have noted subtle psychiatric disease-related phenotypes in the PDE11A knockout mice, including hyperactivity in an open field, and deficits in social behaviors such as social odor recognition memory and social avoidance (Kelly et al., 2010). They
also noted that PDE11 expression in the brain is restricted to the hippocampus, and the enzyme accounts for a small but significant amount of the total PDE activity in ventral hippocampus. It remains to be determined whether these phenotypes are direct results of the loss of PDE11A function or developmental alterations in the mouse model.

1.3.4.4. PDE11A in the immune system

Two recent studies suggest possible roles for PDE11 in the immune system. A genome-wide association scan has linked *PDE11A* variants to risk for asthma (DeWan et al., 2010). Bazhin *et al.* have demonstrated that PDE11 protein is expressed in regulatory and conventional T cells (Treg and Tcon), and has a significant role in the cAMP catabolism in these cells (Bazhin et al., 2010).

1.3.4.5. The function of PDE11 in adrenal glands and its role in the development of adrenocortical tumors and Cushing syndrome

PDE11 is expressed in adrenal glands, and *PDE11A*-inactivating mutations have been identified in patients with adrenocortical tumors and Cushing syndrome, a condition resulting from excess cortisol release from adrenal tumors (Boikos et al., 2008; Carney et al., 2010; Horvath et al., 2006a; Horvath et al., 2006b). PDE11 is the first PDE to be linked with endocrine tumors and to an inherited condition associated with tumor formation. Defects in cAMP signaling have been documented in various endocrine
diseases and adrenal hyperplasia leading to Cushing syndrome (Bourdeau and Stratakis, 2002; Stratakis and Kirschner, 1998).

A genetic defect in the cAMP pathway was first noted in McCune-Albright syndrome (MAS), which is caused by activating mutations in G-protein α subunit, the product of the GNAS gene (Weinstein et al., 1991). Other adrenal diseases, including micronodular adrenocortical hyperplasia (MAH), its pigmented variant, primary pigmented nodular adrenocortical disease (PPNAD), and the multiple endocrine neoplasia syndrome, Carney complex, have been suggested to be caused by germline inactivating mutations of the PRKAR1A gene, which encodes the regulatory subunit of PKA (Groussin et al., 2002; Kirschner et al., 2000; Libe et al., 2011). It is now well-established that alterations in several key components of the cAMP pathway might lead to adrenocortical tumors (Rosenberg et al., 2002a; Stratakis, 2009) (Figure 4).

Recently, PDE11A-inactivating mutations have been reported in patients with several forms of adrenal hyperplasia leading to Cushing syndrome. Three missense (R804H, R867G, and R307X), two frame-shift (171delTfs41X, 1655_1657delTCT/insCCfs15X), and three nonsense mutations in PDE11A gene were found at much higher frequencies in patients with adrenocortical hyperplasia and Cushing syndrome, compared to healthy controls (Horvath et al., 2006a; Horvath et al., 2006b). Adrenal tumor homogenates from these patients have elevated cyclic nucleotide levels and increased
CREB phosphorylation, suggesting that PDE11 plays a major role in controlling cAMP and cGMP levels in these tissues (Horvath et al., 2006a). High levels of PDE11A expression have been found in human adrenal cortex and the cortisol-producing zona fasciculata cells (Horvath et al., 2006a). Boikos et al. have also confirmed the high expression of PDE11A in the adrenal cortex, and demonstrated that the sequence defects in PDE11A gene are associated with a high state of CREB phosphorylation in PPNAD tumors and bilateral adrenocortical hyperplasias that lead to Cushing syndrome (Boikos et al., 2008). A role for PDE11 in the development of adrenal tumors has also been suggested by Libe et al., based on the high frequency of PDE11A sequence variants in patients with Carney Complex (Libe et al., 2011). These studies have demonstrated that inactivating mutations of PDE11A are associated with the development of adrenocortical hyperplasia leading to Cushing syndrome. The link between genetic defects of PDE11A and a condition of excess cortisol release by adrenocortical cells also suggests a role for PDE11 in cortisol production.
Table 2: Sequence comparison of PDE11A with other PDE families

Human PDE11A (NP_058649.3) catalytic domain sequence alignment with other human PDE catalytic domains, using EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

<table>
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<tr>
<th>PDE protein</th>
<th>Catalytic domain sequence identity (%)</th>
<th>Catalytic domain sequence similarity (%)</th>
<th>Accession number</th>
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<tr>
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<td>NP_006195.3</td>
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<td>PDE10A</td>
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<tr>
<td>PDE2A</td>
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<td>60</td>
<td>NP_001137311.1</td>
</tr>
<tr>
<td>PDE4A</td>
<td>33</td>
<td>54</td>
<td>NP_001104777.1</td>
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<tr>
<td>PDE1C</td>
<td>31</td>
<td>51</td>
<td>NP_005011</td>
</tr>
<tr>
<td>PDE9A</td>
<td>29</td>
<td>51</td>
<td>NP_001001570.1</td>
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<td>47</td>
<td>NP_002594.1</td>
</tr>
<tr>
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<td>27</td>
<td>46</td>
<td>NP_002596.1</td>
</tr>
<tr>
<td>PDE3A</td>
<td>21</td>
<td>31</td>
<td>NP_000912.3</td>
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### Table 3: Catalytic and physical properties of PDE11A variants

<table>
<thead>
<tr>
<th>PDE11A Isoform</th>
<th>$K_m$ (μM)</th>
<th>SDS-PAGE M.W. (kDa)</th>
<th>Quaternary structure</th>
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<tbody>
<tr>
<td></td>
<td>cGMP</td>
<td>cAMP</td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>1.6</td>
<td>100</td>
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<tr>
<td>PDE11A3</td>
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<td>0.8</td>
<td>75</td>
</tr>
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<td>0.34</td>
<td>0.45</td>
<td>64</td>
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<td>PDE11A1</td>
<td>0.4</td>
<td>0.45</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 4: Genetic defects of the cAMP signaling pathway in the adrenal glands causing various forms of Cushing syndrome. Binding of ACTH to the G-protein coupled receptor leads to the disassociation of G-protein α subunit (Gα) of the heterotrimeric G-protein to activate adenylate cyclase. cAMP produced by adenylate cyclase binds to the regulatory subunits of PKA, resulting in activation of the catalytic subunits, which activate the CRE-binding (CREB) family of transcription factors. Adrenocortical tumors leading to Cushing syndrome have been associated with genetic defects that cause (1) activation of Gα protein, (2) inactivation of the Protein Kinase A regulatory subunit (R), and (3) inactivation of PDE11A.
Figure 4
1.4. USING A FISSION YEAST-BASED PLATFORM FOR ANALYSES OF MAMMALIAN PDES

Due to the importance of PDEs in various physiological processes and their recognition as valuable therapeutic targets, there have been numerous efforts to develop selective PDE inhibitors. Most of these studies employ in vitro approaches with recombinantly expressed PDE enzymes, or medicinal chemistry and rational drug design studies that are based on crystal structures of the target PDE’s catalytic domain to produce analogs of nonselective PDEs. Although these methods have led to the development of several selective inhibitors, there remains a need for family- and isoform-specific inhibitors of several PDEs, including PDE11A.

In an effort to discover selective inhibitors of mammalian PDEs, we developed a fission yeast-based assay platform that is amenable for high throughput small-molecule screens (HTSs) (Ivey et al., 2008). The screen employs genetically engineered yeast strains whose growth behavior reflects the activity of heterologously-expressed PDEs. In contrast to traditional methods, this approach allows screening of full-length enzymes expressed in eukaryotic cells, under conditions that closely resemble their natural cellular environments. Therefore it is open to the discovery of both active site and allosteric inhibitors that are structurally unrelated to current PDE inhibitors. In addition, by allowing the study of both cAMP- and cGMP-hydrolyzing PDEs, this
platform also allows the characterization of PDE activity in live cells (Demirbas et al., 2011a; Demirbas et al., 2011b).

1.4.1. *S. pombe* glucose/cAMP signaling pathway

Our screening platform makes use of a glucose-repressible *fbp1* (fructose-1,6-biphosphatase) promoter that is regulated by the cAMP signaling pathway and drives the expression of a *ura4* OMP decarboxylase reporter gene. Fission yeast *S. pombe* detects glucose via cAMP signaling (Hoffman, 2005a, b). Most components of this pathway have been identified using mutant strains that are defective in glucose repression of transcription of the *fbp1* gene, which encodes the gluconeogenic enzyme fructose-1,6-biphosphatase (Hoffman and Winston, 1990, 1991) (Figure 5).

The *fbp1-ura4* reporter places uracil biosynthesis under the control of the glucose/cAMP signaling pathway. In response to glucose, adenylate cyclase activation leads to an increase in cAMP production. Cells with high cAMP levels activate PKA to repress *ura4* expression, and cannot grow in medium lacking uracil, but grow in medium containing the pyrimidine analog 5-fluoro-orotic acid (5FOA®), since 5FOA is toxic to the cells that express the *ura4* gene (Figure 6A). Cells with low cAMP levels due to the defects in glucose signaling pathway constitutively express the *fbp1-ura4* reporter, and cannot grow in 5FOA medium (5FOA®) (Figure 6B). Such strains carry mutations that affect the Git3 G-protein coupled receptor (Welton and Hoffman, 2000),
the α, β, and γ subunits (Gpa2, Git5, and Git11) of the heterotrimeric G-protein (Landry and Hoffman, 2001; Landry et al., 2000; Nocero et al., 1994), Git2 adenylate cyclase (Hoffman and Winston, 1991), Pka1 PKA catalytic subunit (Jin et al., 1995), as well as the Git1 adenylate cyclase-binding protein (Kao et al., 2006), Hsp90 (Alaamery and Hoffman, 2008), and Git7 Hsp90 co-chaperone proteins (Schadick et al., 2002). Negative regulators of this pathway were also identified through selections of mutations that restore 5FOA^R growth, such as the cgs1 gene that encodes the PKA regulatory subunit, and the cgs2 gene encoding the only S. pombe PDE (Stiefel et al., 2004; Wang et al., 2005c).

1.4.2. Fission yeast-based HTSs for small-molecule inhibitors of mammalian PDEs

In order to discover novel inhibitors of mammalian PDEs, the screening platform that has revealed genetic regulators of the cAMP signaling pathway was coverted into an assay system to identify chemical regulators of heterologously-expressed PDEs in yeast cells. The first screens for PDE4 and PDE7 inhibitors employed strains with mutations affecting either the Git3 G-protein coupled receptor, or a subunit of the heterotrimeric G-protein, which reduce cAMP synthesis in response to glucose, conferring 5FOA^S growth (Alaamery et al., 2010; Ivey et al., 2008). Inhibition of PDE activity in these cells restores 5FOA^R growth, allowing detection of PDE inhibitor compounds via a simple growth assay in 5FOA medium.
The strains suitable for the initial HTSs were created based on ability of the heterologous PDE to reduce intracellular cAMP levels. For a robust PDE inhibitor screen, the PDE must lower cAMP levels sufficiently to create a 5FOA\(^5\) growth phenotype that will be converted to 5FOA\(^R\) growth upon inhibition of the enzyme. In order to have greater over PKA activity, we created strains that lack adenylate cyclase activity by deleting the git2 gene. These strains allow the regulation of PKA activity by adding exogenous cAMP to the medium (Demirbas et al., 2011a; Demirbas et al., 2011b). In addition, since we have shown that like in the budding yeast, exogenous cGMP also activates PKA in *S. pombe* (Cytrynska et al., 1999; Demirbas et al., 2011a), this system can also detect inhibition of cGMP-specific and dual-specific PDEs via the increase in the cGMP amount required to confer 5FOA\(^R\) growth (Demirbas et al., 2011b). For the purposes of profiling the substrate-specificity and inhibitor-sensitivity of mammalian PDEs, we have constructed strains expressing variants from 10 of the 11 PDE families (with the exception of PDE6 family), all of which are suitable for PDE characterization and inhibitor screens (Table 4).

1.5. **AIM OF THE STUDY**

The aim of this study is to identify the first PDE11-specific inhibitors, using a yeast-based high throughput screen. These compounds will be extremely beneficial as research tools to elucidate PDE11 function in various tissues and as leads for developing novel therapeutics for the treatment of several diseases that have been linked to PDE11.
**Figure 5: S. pombe senses glucose via a cAMP signaling pathway.** Glucose binding to the Git3 G-protein coupled receptor leads to the activation of Git2 adenylate cyclase by the heterotrimeric G-protein (Gpa2, Git5, and Git11). cAMP produced by adenylate cyclase activates PKA by disrupting the interaction of PKA regulatory subunits with the catalytic subunits. Intracellular cAMP levels are negatively regulated by the activity of Cgs2 PDE. The components of this pathway were discovered using the *fbp1-ura4* reporter that is repressed by PKA upon an increase in cAMP levels.
Figure 6: The *fbp1-ura4* reporter allows monitoring intracellular cAMP levels via the growth phenotype of cells in 5FOA medium. (A) Wild-type cells respond to high glucose levels by activating adenylate cyclase to increase cAMP levels. This leads to repression of the *fbp1-ura4* reporter and confers 5FOA resistant growth. (B) Cells with a defect in the glucose sensing pathway have low cAMP levels, which fail to activate PKA. This leads to expression of the *fbp1-ura4* reporter and 5FOA-sensitivity.
Figure 6

A

Glucose → AC → ATP → cAMP → PKA

Glucose → PDE → cAMP → 5'AMP → PKA

PKA → ura4

5FOA resistant

B

Glucose

Defect in glucose sensing pathway

AC → ATP → cAMP → PKA

AC → PDE → cAMP → 5'AMP → PKA

PKA → ura4

5FOA sensitive
CHAPTER TWO

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Yeast strains and growth media

The yeast strains used in this study are listed in Table 4. All of the strains possess
$fbp1::ura4^+$ and $ura4::fbp1-lacZ$ reporters, which are translational fusions integrated at
the $fbp1^+$ and $ura4^+$ loci, respectively (Hoffman and Winston, 1990). Strains with
mammalian PDEs had the following inserts (mammalian PDE nomenclature utilizes a
number to identify the PDE family, followed by a letter to indicate a particular gene
within that family, followed by another number to indicate a splice variant when
multiple splice variants are known): PDE1B1 (RefSeq: NM_000924), mouse PDE2A2
(RefSeq: NM_001008548.3), human PDE3A1 (RefSeq: BC117371.1), human PDE4A1
(RefSeq: U68532), bovine PDE5A1 (RefSeq: NM_174417.2), human PDE7A1 (RefSeq:
NM_002603), murine PDE8A1 (RefSeq: ), human PDE9A5 (RefSeq: NM_001001570.1),
human PDE10A1 (RefSeq: AF127479.1), human PDE11A4 (RefSeq: NM_016953.3).

Yeast cells were grown and maintained in yeast extract agar (YEA) and yeast extract
liquid (YEL) media supplemented with 100 mg/L adenine. Defined medium EMM (MP
Biochemicals) was supplemented with required nutrients at 75 mg/L, except for L-
leucine, which was at 150 mg/L. Sensitivity to 5-fluoro-orotic acid (5FOA) was
determined on SC solid medium containing 0.4 g/L 5FOA and 8% glucose (Hoffman and
Winston, 1990). Liquid 5FOA growth medium was the same as the solid medium, but without agar. Cells were grown at 30°C.

2.1.2. Bacterial strains and growth media

ElectroTen-Blue electroporation competent cells (Agilent Technologies – Stratagene) were used as *Escherichia coli* host strain for plasmid amplification. Bacterial cells were grown in Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) and transformants were selected on LB media with 100 mg/L ampicillin. Cells were grown at 37°C.

2.1.3. Mammalian cells and culture media

Human NCI-H295R cells were obtained from the American Type Culture Collection (ATCC) and were maintained in an equal mixture (vol/vol) of Dulbecco’s Modified Eagle’s (DMEM) and Ham’s F12 Media (DME-F12) containing insulin (1 µg/ml), transferrin (1 µg/ml), selenium (1 ng/ml), linoleic acid (1 µg/ml), BSA (1.25 mg/ml) added in the form of ITS plus mix (BD Biosciences), 2.5% Nu-Serum (BD Biosciences), and antibiotics. To eliminate the influence of hormones in the serum, cells were switched to media containing no serum for the experiments. HeLa and MDA-MB-231 cells were gifts from David Sabatini and were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS. Cells were maintained at 37°C in 5% CO₂.
Table 4: Yeast strains used in the study

<table>
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<th>PDE in the strain</th>
<th>Strain ID</th>
<th>Genotype</th>
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<td>CHP1403</td>
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<td>CHP1249</td>
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<td>CHP1223</td>
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<td>CHP1189</td>
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<td>$h^+$ $fbp1::ura4+$ $ura4::fbp1-lacZ$ $leu1-32$ $his7-366$ $pap1\Delta::ura4-$ $cgs2::PDE11A$ $git2-2::his7+$</td>
</tr>
</tbody>
</table>
2.1.4. Small molecules

Small molecules were purchased from ChemDiv, ChemBridge, LifeSciences, Maybridge, and Enamine, and 10-50 mM stock solutions were prepared by dissolving in DMSO.

2.2. METHODS

2.2.1. 5FOA growth assays

Assays were performed using strains that express human PDE1B1, PDE3A1, PDE4A1, PDE7A1, PDE9A5, PDE10A1, and PDE11A4; murine PDE2A2 and PDE8A1; and bovine PDE5A1. The pregrowth and assay conditions were optimized for each strain (the optimal conditions are listed in Table 5). Prior to the start of 5FOA growth assays, cells were grown to exponential phase under conditions that repress the *fbp1-ura4* reporter (Table 5). Cells were collected by centrifugation and inoculated into 50 μl 5FOA medium in two to four replicate wells in a 384-well microtiter plate at the optimal starting concentration for the strain. The plates were incubated at 30°C for 48 h in a container with moist paper towels to reduce evaporation in the wells. After vortexing the plates using a microtiter dish vortexer, optical densities (OD) were measured at 600 nm using a microtiter plate reader. All assays were performed at least three times.

Dose response profiling of compounds was performed in 5FOA medium with compound concentrations in a range of from 100 μM to 0.101 μM, by making 2/3 dilutions in 18 consecutive columns. Cells were added in 25 μl to wells containing 25 μl of 5FOA medium with 2x compound concentration, to achieve 50 μl final volume. Plate
incubations and OD measurements were performed as described in the 5FOA growth assay procedure.

2.2.2. High throughput screening (HTS)

HTS was performed at the ICCB-Longwood Screening Facility of Harvard Medical School. Yeast cells that express human PDE11A4 were grown in EMM medium with 0.25 mM cAMP for 24 h to $\sim 10^7$ cells/ml. The screening 5FOA medium is SC-based and contains 0.4 g/L 5FOA. 25 ml 5FOA medium was transferred into duplicate 384-well flat, clear-bottom microtiter dishes and 100 nl of compounds (from stock solutions of generally $\sim 10$-15 mM) were pinned into the wells. Cells were collected by centrifugation, resuspended in 5FOA medium with 120 $\mu$M cGMP, and 25 $\mu$l was transferred into each well at an initial cell density of 0.75x10^5 cells/ml. Control plates consisted of positive control cultures containing 25 $\mu$M and 40 $\mu$M BC76, and negative control cultures containing 0.2% DMSO. Each screening plate included internal positive and negative control wells. Plates were incubated at 30°C for 48 h in a closed container with moist paper towels to prevent evaporation. ODs of the cultures were measured at 600 nm. In cherry-picking experiments, 100 nl compounds were added using pocket tips instead of steel pin arrays.
Table 5: Optimal conditions used for each strain in 5FOA growth assays

<table>
<thead>
<tr>
<th>PDE</th>
<th>Pregrowth supplement</th>
<th>Cyclic nucleotide in 5FOA medium</th>
<th>Cell density (x10^5 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1B</td>
<td>2.5 mM cAMP</td>
<td>15 µM cAMP</td>
<td>0.5</td>
</tr>
<tr>
<td>PDE2A</td>
<td>0.25 mM cAMP</td>
<td>15 µM cAMP</td>
<td>2</td>
</tr>
<tr>
<td>PDE3A</td>
<td>5 mM cAMP</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>PDE4A</td>
<td>5 mM cAMP</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>PDE5A</td>
<td>0.25 mM cAMP</td>
<td>200 µM cGMP</td>
<td>2</td>
</tr>
<tr>
<td>PDE7A</td>
<td>2.5 mM cAMP</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>PDE8A</td>
<td>0.5 mM cAMP</td>
<td>40 µM cAMP</td>
<td>0.5</td>
</tr>
<tr>
<td>PDE9A</td>
<td>0.25 mM cAMP</td>
<td>600 µM cGMP</td>
<td>2</td>
</tr>
<tr>
<td>PDE10A</td>
<td>0.25 mM cAMP</td>
<td>15 µM cAMP</td>
<td>2</td>
</tr>
<tr>
<td>PDE11A</td>
<td>0.25 mM cAMP</td>
<td>60 µM cGMP</td>
<td>0.75</td>
</tr>
</tbody>
</table>
2.2.3. Data analysis

$Z'$-factor testing was done at the start of the HTS and in every assay during the HTS process to check the quality of each plate, using results of the internal controls. $Z'$-factor of assays were calculated as $1 - \left(3(\sigma_p + \sigma_n)/|\mu_p - \mu_n|\right)$, where $\sigma_p$ and $\sigma_n$ are the standard deviations, and $\mu_p$ and $\mu_n$ are the means of the positive and negative controls. An assay with a $Z'$-factor $>0.5$ is considered sufficiently robust for HTS. Within a screen, individual wells are assigned a $Z$ score, representing the number of standard deviations above or below the mean of the negative control wells. Duplicate $Z$ scores for each compound are plotted onto a grid and projected perpendicularly to the diagonal. A Composite $Z$ score is the distance from this point on the diagonal to the origin.

2.2.4. In vitro PDE enzyme assays

In vitro enzyme assays were conducted via the $\text{Ba(OH)}_2$ precipitation method of Wang et al. (Wang et al., 2005a), using recombinant human PDE1C, PDE3B, PDE5A1, PDE6C, PDE8A, PDE9A2, PDE10A1, PDE11A4, and rat PDE2A (BPS Bioscience Inc.), human PDE7A (BIOMOL International), and human PDE4A10 enzymes (gift from Hengming Ke). The substrate concentrations used for each enzyme are as follows: 100 nM cGMP (PDE1C), 1 $\mu$M cGMP (PDE2A), 30 nM cGMP (PDE3B), 625 nM cAMP (PDE4A), 500 nM cGMP (PDE5A), 1.7 $\mu$M cGMP (PDE6C), 15 nM cAMP (PDE7A), 10 nM cAMP (PDE8A), 70nM cGMP (PDE9A), 30 nM cAMP (PDE10A), 100 nM cGMP (PDE11A). Inhibitor concentrations that reduce enzyme activity by 50% (IC$_{50}$) are presented. The values are
means of at least three independent experiments. Substrate concentrations were \( \leq 0.1*K_m \) for each enzyme, thus \( IC_{50} \) values approximate the \( K_i \) values.

### 2.2.5. cAMP assays and immunoblot analysis

cAMP assays and immunoblot analysis were performed using NCI-H295R, HeLa, and MDA-MB-231 cells. ~90% confluent cells in 12-well dishes were incubated in serum-starved media for 1 h, then in 0.5 ml serum-starved media with 20 mM compounds or 0.2% DMSO for 2 h in the absence or presence of 10 \( \mu \)M forskolin (Sigma Aldrich). Media was collected and cAMP levels were measured as described (Rainey et al., 1993), using a cAMP ELISA kit (Enzo). cAMP levels were normalized to protein content, which was measured using a BCA Protein Assay Kit (Pierce). Immunoblot analysis of protein lysates was performed as described (Sarbassov et al., 2006), using phospho-CREB(9198), Akt(4685), or CREB(9197) primary antibodies (Cell Signaling Technology) and goat anti-rabbit IgG-HRP secondary antibody (sc-2030, Santa Cruz).

### 2.2.6. Cortisol assays

H295R cells were treated with compounds as described in the cAMP assays, for 24 h. Media was collected and cortisol content was quantified using a Cortisol EIA Kit (Oxford Biomedical Research). Cortisol levels were normalized to protein concentrations in cell extracts, as described for the cAMP and immunoblot experiments.
2.2.7. Quantitative RT-PCR

Total cellular RNA was isolated using RNeasy minikits (QIAGEN). cDNA was synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase with random hexamers. PDE11A expression was determined by a SYBR Green Real Time PCR assay (ABI) using PDE11A-specific primers (5’ TGGAGTGGATTGATAGCATCTG 3’ and 5’ TTTGGTAGCTCTTCCCAC 3’). Expression levels were normalized to RPLP0 expression (IDT).
CHAPTER THREE

IDENTIFICATION OF PDE11-SELECTIVE INHIBITORS USING A YEAST-BASED HIGH THROUGHPUT SCREEN

3.1. DEVELOPMENT OF A YEAST-BASED SCREENING PLATFORM FOR PDE11 INHIBITORS

I used the fission-yeast based PDE assay to develop a HTS for small-molecule inhibitors of the human PDE11A4 enzyme. The screening strain (CHP1224) was previously generated by replacing the open reading frame of the only S. pombe PDE gene, cgs2+, with a human PDE11A4 cDNA via homologous recombination (Table 4) (Demirbas et al., 2011b). This strain has a deletion of the git2 gene that encodes adenylate cyclase. This allows fbp1-ura4 reporter expression to be regulated by exogenous cAMP and cGMP that is added to the 5FOA growth medium. CHP1207 strain, which lacks both adenylate cyclase and PDE activity, responds to low levels of exogenous cyclic nucleotide to activate PKA and thus repress ura4 expression, conferring 5FOA<sup>R</sup> growth. Cells that express PDE11 hydrolyze the exogenously added cyclic nucleotide and remain 5FOA<sup>S</sup> (Figure 7A), while the addition of a PDE inhibitor confers 5FOA<sup>R</sup> growth (Figure 7B).
**Figure 7: Schematic of the PDE11 inhibitor screen.** PDE11 screening strain (CHP1224) possesses the *fbp1-ura4* reporter, whose expression can be repressed by PKA that is activated by the addition of cGMP to the 5FOA growth medium. cGMP hydrolysis by PDE11 allows *ura4* expression, producing a 5FOA\(^5\) phenotype (A). PDE11 inhibition elevates intracellular cGMP levels to confer 5FOA\(^R\) growth (B).
Figure 7

A

\begin{center}
\begin{tikzpicture}
    \node (cGMP) at (0,0) {cGMP};
    \node (ura4) at (1.5,0) {\textit{ura4}}; \node [draw, below=of ura4] {5FOA sensitive};
    \node (GMP) at (3,0) {GMP};
    \draw[->] (cGMP) -- (ura4);
    \draw[->] (ura4) -- (GMP);
    \draw[->] (ura4) -- (GMP);
    \draw[->] (ura4) -- (GMP);
\end{tikzpicture}
\end{center}

B

\begin{center}
\begin{tikzpicture}
    \node (cGMP) at (0,0) {cGMP};
    \node (ura4) at (1.5,0) {\textit{ura4}}; \node [draw, below=of ura4] {5FOA resistant};
    \node (PDE11) at (3,0) {PDE11};
    \node (PDE11 inhibitor) at (3,1) {PDE11 inhibitor};
    \draw[->] (cGMP) -- (PDE11);
    \draw[->] (PDE11) -- (GMP);
    \draw[->] (PDE11 inhibitor) -- (PDE11);
    \draw[->] (PDE11 inhibitor) -- (PDE11);
\end{tikzpicture}
\end{center}
The activity of PDE11 for cAMP and cGMP hydrolysis when expressed in fission yeast was analyzed by comparison of the cyclic nucleotide requirements of the strain that expresses PDE11 (CHP1224) with those of the strain that lacks PDE activity (CHP1207). Cells expressing PDE11A4 require more cyclic nucleotide in the growth medium to achieve 5FOA resistance (Figure 8), indicating that the expressed enzyme is functionally active in yeast. The PDE11 enzyme produces a greater cGMP requirement as compared to the cAMP requirement in *S. pombe*, therefore HTS conditions were optimized for using cGMP in the 5FOA growth medium.

### 3.2. Optimization of the Yeast-Based HTS for PDE11 Inhibitors

The optimal conditions for a robust PDE inhibitor HTS should provide the largest difference in the growth of cells when the PDE is active versus inhibited by a compound, while keeping the well-to-well variability low. In order to determine the optimal conditions for the screen, a previously-identified nonselective inhibitor, BC76 (Demirbas et al., 2011b) was used as a positive control for PDE11 inhibition (Figure 9A). Since compound concentrations in the screening libraries are 20-30 µM, BC76 was used at 25 µM. The screening conditions were optimized using a 384-well format. Assay development involved optimizing: (1) pre-assay growth conditions that establish repression of the *ura4* reporter, which is maintained by a PDE inhibitor during the 48-h incubation period of the screen, (2) initial cell density, and (3) cGMP concentration in the screening medium that confers saturated growth only in the presence of BC76.
5FOA growth assays with the PDE11-expressing strain demonstrated that pre-growth of cells in complete medium with 0.25 mM cAMP, and screening in 5FOA medium with 60 µM cGMP using an initial cell density of 0.75x10^5 cells/ml allow cells to grow to saturation (optical density (OD) ~1.1) after 48 h at 30°C in the presence of 25 µM BC76, but fails to promote growth in the absence of BC76 (OD ~0.2) (Figure 9B). Thus, these conditions were selected to be used for a robust PDE11 inhibitor HTS.

Statistically, the robustness of screening conditions is determined by a Z'-factor analysis (Zhang et al., 1999). The Z'-factor is based on the difference between the positive controls (in presence of a compound known to inhibit the target PDE) and negative controls (in presence of DMSO, the solvent for the compounds in screening libraries) as a function of the variability among the datapoints. Z'-factor equals 1- (3(σ_p + σ_n)/|µ_p - µ_n|), where σ_p and σ_n are the standard deviations, µ_p and µ_n are the means of the positive and negative control. An assay with a Z'-factor of greater than 0.5 is considered to be sufficiently robust for high throughput screening. The optimized screening conditions were tested at the ICCB-Longwood Screening Facility, and produced Z'-factors of 0.7-0.9, indicative of a robust screen.
Figure 8: cAMP and cGMP hydrolysis by the PDE11-expressing strain. 5FOA growth assays with strains lacking PDE activity (cgs2-2 is a frameshift allele of the only S. pombe PDE gene (Wang et al., 2005b)) or expressing human PDE11A4 were performed in 0-2 mM cAMP and cGMP. Values are mean of three experiments (with three wells per condition in each experiment) ±S.E.M. (The error bars are smaller than the symbols in datapoints where they cannot be observed).
Figure 8

A

Optical Density

PDE11

[50] cgs2-2

[cAMP] (mM)

0.00 0.05 0.10 0.15 0.20

B

Optical Density

PDE11

[50] cgs2-2

[cGMP] (mM)

0.00 0.05 0.10 0.15 0.20
Figure 9: Optimization of the HTS conditions for PDE11 inhibitor screen. (A)
Structure of the positive control compound BC76. (B) 5FOA growth assays with the
PDE11-expressing strain in medium containing either 0.2% DMSO or 25 μM BC76, with
varying concentrations of cGMP (0-2 mM). The vertical line indicates the cGMP
concentration that produces the maximum difference in average OD between the
DMSO-treated and BC76-treated cultures. Values are mean of three experiments (with
three wells per condition in each experiment) ±S.E.M.
Figure 9

A

BC76

B

Screening Dose

Optical Density

- PDE11
- PDE11+BC76

[cGMP] (mM)
3.3. PRIMARY HIGH THROUGHPUT SCREEN FOR PDE11 INHIBITORS

3.3.1. Pilot screen with the Known Bioactives Collection

The HTS for PDE11 inhibitors was performed at the ICCB-Longwood Screening Facility of Harvard Medical School. In the screening process (Figure 10A), 25 µl 5FOA medium was transferred into duplicate 384-well microtiter dishes, and 100 nl compounds from the library plates were pinned into the wells. This was followed by collection of the PDE11-expressing cells, which had been grown to ~10^7 cells/ml density in the appropriate pre-assay medium, and resuspension in 5FOA medium with 120 µM cGMP. 25 µl of these cells were then transferred into each well at the optimal initial cell density to produce a final cGMP concentration of 60 µM. Compound stock solutions in different libraries were generally ~10-15 mM, resulting in ~20-30 µM final compound concentration in the screening cultures. Control plates consisted of positive control cultures containing 25 µM and 40 µM BC76, and negative control cultures containing 0.2% DMSO. Each screening plate also included internal positive and negative control wells (Figure 10B). Another positive control plate with 1 mM cAMP in 5FOA medium was used in each experiment. At the end of a 48-h incubation period in 5FOA medium, growth in each well was assessed by measuring optical density (OD).

Before starting the HTS, ICCB recommends a pilot screening of the Known Bioactives Collection, especially for cell-based screens. This collection consists of ~4,770 well-characterized compounds that affect a variety of biological pathways, including FDA-
approved drugs and molecules that have a history of use in NIH human clinical trials. As a pilot screen, the Known Bioactives Collection was screened and results were analyzed by assigning a Z score to individual wells, which represents the number of standard deviations above or below the mean of the negative control wells. Duplicate Z scores for each compound were plotted onto a grid to examine the reproducibility of the results. In this screen, the Z scores for the negative and positive controls were ~0-2 and ~50-80, respectively. Only one compound, Br-cAMP, an analog of cAMP that is a positive control in this screen, appeared as a hit with Z scores of ~140 in both replicates (Figure 11). The absence of other hits within this collection was not surprising, since there are no known potent PDE11 inhibitors available.

3.3.2. HTS with commercial libraries

Since satisfactory results were obtained from the pilot screen, I proceeded to the additional commercial libraries, following ICCB’s recommended order, which is based on the freshness of the stock plates (more recently prepared libraries were screened first, with the exception of ChemDiv6 that is only allowed to be screened once an assay is demonstrated to be of very high quality, based on Z’-factors of the assays) (Table 6). 198,382 compounds were screened in duplicate in the primary HTS. The Z scores of the duplicate wells are presented in Figure 12.
Figure 10: High throughput screening process. (A) 5FOA medium was transferred into 384-well microtiter dishes using a WellMate liquid handler. 100 nl compound was added to each well from stock chemical libraries using a steel pin array. Cultures of PDE11-expressing cells in the screening medium were then added into the wells. Plates were incubated at 30°C for 48 hours, and OD of the cultures were determined by reading the absorbance of each well at 600 nm, using an EnVision Plate Reader. (B) Example readout of a screening plate. The last column contains internal positive and negative controls. The colors of the wells indicate their absorbance values, increasing from blue to red. Hit compounds were identified based on the higher OD of the cultures, while compounds that are toxic to yeast caused lower OD values compared to the negative controls.
Figure 10

A

384-well microtiter dishes

Transfer 5FOA medium into plates

Compounds are pinned

Read OD of cultures

Incubate at 30°C for 48 h

Transfer yeast cells into plates

B

Internal positive control (25 μM BC76)

Internal negative control (0.2% DMSO)
**Figure 11: Results of the Known Bioactives screen.** Scatter plot represents Z scores for duplicate wells pinned with 0.2% DMSO (negative controls; *dark blue circles*), 25 µM BC76 (positive controls; *red circles*), or screening compounds (*light blue circles*). *Grey circles* represent empty wells that were only filled with cells in 5FOA medium. Br-cAMP (with Z scores ~140 in both replicates) was the only hit in this pilot screen.
Figure 11
Table 6: Screened small-molecule libraries by recommended order of ICCB-Longwood Screening Facility.

<table>
<thead>
<tr>
<th>Library name</th>
<th># comp</th>
<th>Plate numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Known Bioactives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomol ICCB Known Bioactives 3 – high conc</td>
<td>480</td>
<td>1989-1990</td>
</tr>
<tr>
<td>Ninds Custom Collection</td>
<td>1,040</td>
<td>1920-1923</td>
</tr>
<tr>
<td>Prestwick 1 Collection</td>
<td>1,120</td>
<td>1568-1571</td>
</tr>
<tr>
<td>Microsource 1 – US Drug Collection</td>
<td>1,040</td>
<td>2091-2094</td>
</tr>
<tr>
<td>NIH Clinical Collection 1</td>
<td>450</td>
<td>2051-2052</td>
</tr>
<tr>
<td>Biomol 4 – FDA Approved Drug Library</td>
<td>640</td>
<td>2089-2090</td>
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<tr>
<td><strong>Most Recently Plated Commercial Compounds</strong></td>
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<td></td>
</tr>
<tr>
<td>Asinex 1</td>
<td>12,378</td>
<td>1671-1706</td>
</tr>
<tr>
<td>ChemBridge 3</td>
<td>10,560</td>
<td>1577-1606</td>
</tr>
<tr>
<td>ChemDiv 4</td>
<td>14,677</td>
<td>1607-1648</td>
</tr>
<tr>
<td>Enamine 2</td>
<td>26,576</td>
<td>1715-1790</td>
</tr>
<tr>
<td>Life Chemicals 1</td>
<td>3,893</td>
<td>1649-1660</td>
</tr>
<tr>
<td>Maybridge 5</td>
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<td>1661-1670</td>
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<tr>
<td><strong>Other Recently Plated Commercial Compounds</strong></td>
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<tr>
<td>ChemDiv 3</td>
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<td>ChemDiv 2</td>
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<td>1369-1393</td>
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<tr>
<td>Enamine 1</td>
<td>6,004</td>
<td>1394-1411</td>
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<tr>
<td>I.F. Lab 2</td>
<td>292</td>
<td>1459</td>
</tr>
<tr>
<td>Maybridge 2</td>
<td>704</td>
<td>1303-1304</td>
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<tr>
<td>Maybridge 3</td>
<td>7,639</td>
<td>1431-1452</td>
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<tr>
<td><strong>The Remainder of the Collection</strong></td>
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<tr>
<td>Bionet 1</td>
<td>4,800</td>
<td>568-582</td>
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<tr>
<td>CEREPI</td>
<td>4,800</td>
<td>526-540</td>
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<td>ChemDiv1 (Combilab and Int'l)</td>
<td>28,864</td>
<td>587-668</td>
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<td>ChemDiv Antimitotic Collection</td>
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<td>ChemDiv6</td>
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<td>1795-1919</td>
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</table>
Figure 12: Summary of HTS data as Z scores. Scatters plot represents Z scores for duplicate wells pinned with 0.2% DMSO (negative controls; *pink circles*), 25 μM BC76 (positive controls; *yellow circles*), or screening compounds (*black circles*).
Figure 12
Modest variations were observed in the growth of cells on different experiments during the screen, as judged by differences in the absorbance values of positive and negative controls on distinct days of the experiment. This is most likely due to temperature fluctuations that the cells encountered while being carried to the screening facility and/or problems with the incubators. However, the growth of cells in both positive and negative control wells, as well as in the screening wells were affected from these temperature variations equally. Figure 13 demonstrates that the Z score corresponding to a particular absorbance value varied in different experiments. However, since Z scores are assigned based on both negative control absorbances and variations among individual wells, they provided consistent interpretation of the results in the overall HTS process.

Candidate hit compounds were defined based on their Composite Z scores, which reflect both the level of growth stimulation by each compound and the reproducibility of the replicate assays. Composite Z scores are calculated by projecting the duplicate Z scores of each compound perpendicularly to the diagonal of the scatter plot and determining the distance from this point on the diagonal to the origin. The distribution of Composite Z scores in the PDE11 screen demonstrates that a very small portion of the screened compounds had scores >20, indicating a robust HTS (Figure 14A). The Composite Z scores for BC76 positive control wells ranged from 26 to 148 (Figure 14B).
**Figure 13: Results of the HTS as absorbance values.** Top panel, scatter plot representing absorbance values (ODs) for duplicate wells pinned with 0.2% DMSO (negative controls; *pink circles*), 25 µM BC76 (positive controls; *yellow circles*), or screening compounds (*black circles*). Bottom panel, scatter plot of the absorbance values (x-axis) versus Z scores (y-axis) for one of the replicates in the HTS.
Figure 13

Absorbance (RepB)

Absorbance (RepA)

- Yellow: Positive control (BC76)
- Magenta: Negative control (DMSO)
- Black: Screened compounds

Z score (RepB)

Absorbance (RepB)
Figure 14: Composite Z score results of the HTS. (A) Composite Z score distribution of the screened compounds. The height of each bar represents the number of screened compounds displaying the corresponding Composite Z score in the x-axis. The x-axis is binned into 537 bins with intervals of 0.4. Compounds with Composite Z scores >20 are defined as hits. (B) Composite Z score (y-axis) of each well, pinned with 0.2% DMSO (negative controls; pink circles), 25 µM BC76 (positive controls; yellow circles), or screening compounds (black circles), grouped by compound plates along the x-axis (each point represents one compound, each vertical column represents one compound plate).
Figure 14

A

Counts

Composite Z

B

Composite Z score

Compounds

- Yellow: Positive control (BC76)
- Red: Negative control (DMSO)
- Black: Screened compounds
Test compounds that promoted significant 5FOA<sup>R</sup> growth were grouped by their Composite Z scores as either strong (>35), moderate (26-35), or weak (20-26) hits (Figure 15). With these definitions, 422 compounds were defined as strong, 302 compounds as moderate, and 419 compounds were defined as weak hits, resulting in 1143 total hits. While the overall frequency of strong and moderate hits in this screen was 0.36%, this frequency varied significantly among the compound libraries (Figure 15A). Of the large libraries screened the highest frequencies of hits were observed in the Actimol TimTec 1 (0.8%; 68 hits from 8,518 compounds), Chembridge 3 (0.7%; 74 hits from 10,560 compounds), and ChemDiv 6 (0.64%; 283 from 44,000 compounds) libraries. In contrast, the lowest frequency of hits was observed in the ChemDiv 1 library (0.04%; 6 hits from 16,544 compounds), which is one of the oldest libraries screened.

3.4. VALIDATION OF PRIMARY SCREEN HITS AND SELECTION OF PDE11-SPECIFIC CANDIDATES BY CHERRY-PICK EXPERIMENTS

3.4.1. Selection of compounds for cherry-pick experiments

The first step toward identification of PDE11-specific compounds among the 1143 primary screen hits was confirming the hits by rescreening the PDE11 strain. Also, an initial elimination of non-specific inhibitors was necessary in order to have a small list of high-priority candidates for secondary assays. For these purposes, 595 compounds (0.3% of the total compounds screened) were rescreened against PDE11 for
verification, and against yeast strains expressing either of the two most structurally-similar PDEs to PDE11 (PDE5 and PDE10) for elimination of nonselective inhibitors and compounds that stimulate cell growth via PDE-independent mechanisms, a step called “cherry-picking”. These top 595 candidates were selected by taking advantage of data from previous HTSs by our lab for PDE4, PDE7, and PDE8 inhibitors at the ICCB Screening Facility and The Broad Institute, which screened some, but not all, of the same compounds in the PDE11 HTS. From the 1143 PDE11 hits, 198 compounds were excluded as they produced Composite Z scores >13 or average absorbance >3 in the PDE8 screen. Another 146 common hits with the PDE4 and PDE7 screens were also excluded from further consideration (Figure 16). In addition, compounds with average absorbance <0.195 in the PDE11 screen were not included in cherry-picking. Finally, Br-cAMP, which was also a common hit with the PDE8 screen, was excluded. This approach yielded 595 compounds to be tested in cherry-pick screens against PDE11-, PDE5-, and PDE10-expressing strains.

In the primary HTS, 11 compounds performed better than all positive controls, with Composite Z scores >148 (Table 7). These compounds all produced high Composite Z scores in PDE4, PDE7, or PDE8 screens as well, therefore were not selected for cherry-pick experiments. They are likely to be nonselective PDE inhibitors, or compounds that promote cell growth via PDE-independent mechanisms.
Figure 15: Classification of hits based on Composite Z score in the HTS. (A)

Composite Z score (y-axis) of screened compounds grouped by compound plates along the x-axis (each point represents one compound, each vertical column represents one compound plate). (B) Scatter plot for Z scores of screened compounds. Values for each of the two replicates are plotted on the x- and y-axis. Strong hits (blue), moderate hits (red), and weak hits (yellow). Compounds with Composite Z score >35 were defined as strong, 26-35 were defined as moderate, and 20-26 were defined as weak hits.
Figure 15

A

B

- Strong
- Moderate
- Weak
- No hit
Figure 16: Selection of compounds for cherry-pick screens. (A) Bar chart representing the classification of 1143 hits. Strong hits (blue), moderate hits (red), and weak hits (yellow). 146 PDE4/7 hit compounds and 198 PDE8 hit compounds were eliminated, together with Br-cAMP. 203 compounds that produced average OD <0.195 in the PDE11 HTS were also excluded for being weak candidates. Among the 595 compounds selected for cherry-pick experiments, 241 were strong, 210 were moderate, and 144 were weak hits in the PDE11 primary HTS.
Figure 16

A

Bar graph showing the count of different categories:
- Cherry-picks: 241
- PDE4/7 hit: 144
- PDE8 hit: 114
- Weak PDE11 hit: 184
- cAMP: 1

Legend:
- Red: Strong hit
- Blue: Moderate hit
- Yellow: Weak hit

B

Pie chart showing the distribution of different categories:
- Cherry-picks: 595
- PDE8 hits: 198
- PDE4/7 hits: 146
- Weak PDE11 hits: 203
- cAMP: 1
3.4.2. Cherry-pick experiments to confirm PDE11 hits and eliminate nonselective inhibitors

The 595 candidates were rescreened against the PDE11-expressing strain for confirmation, and against strains that express PDE5 and PDE10 for elimination of non-specific inhibitors. Cherry-pick experiments involved transferring 100 nl of each compound into 3 plates (in rows C-N, columns 3-22 in plates 1&2 and columns 3-12 in plate 3) using pocket tips, which were then screened against PDE11-, PDE5-, and PDE10-expressing strains. The last column of each plate was pinned with positive control (rows A-H) (25 µM BC76, which inhibits all of the PDEs in the experiment), and negative control (rows I-P) (0.2% DMSO). 163 compounds produced an optical density >0.167 for the PDE11 strain. Among these, 126 compounds that yielded Z scores >8.5 were considered to provide significant growth stimulation, and were accepted as validated PDE11 hits (Figure 17). While data from previous screens allowed avoiding many nonselective and off-target hits, a small group of these primary hits were found to inhibit PDE5 and PDE10. Compounds that produced optical density >0.2 for the PDE5 strain (15 compounds) were defined as PDE5 hits, and >0.195 for the PDE10 strain (25 compounds) were defined as PDE10 hits (Figure 17). Out of the 595 compounds, 6 were hits only against PDE10, 3 were hits only against PDE5, and 2 were hits for all three PDEs (Table 8). 99 of the validated hits were specific for PDE11 inhibition in the cherry picking experiments (Figure 18). Moderate and strong PDE5- and/or PDE10-hits were considered as non-specific.
**Table 7: Strong hits that outperformed all positive controls in the HTS**

<table>
<thead>
<tr>
<th>ICCB plate/well #</th>
<th>Structure</th>
<th>Ave OD</th>
<th>Comp Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1911-N1</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.6725</td>
<td>198.78</td>
</tr>
<tr>
<td>1499-P10</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>0.6765</td>
<td>197.2</td>
</tr>
<tr>
<td>1597-A7</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>0.723</td>
<td>196.1</td>
</tr>
<tr>
<td>1529-D2</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>0.716</td>
<td>184.7</td>
</tr>
<tr>
<td>1509-A14</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>0.623</td>
<td>180.1</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>Value</td>
<td>Angle</td>
</tr>
<tr>
<td>------------</td>
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<td>--------</td>
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</tr>
<tr>
<td>1602-N14</td>
<td><img src="image" alt="Structure" /></td>
<td>0.629</td>
<td>167.6</td>
</tr>
<tr>
<td>1785-B7</td>
<td><img src="image" alt="Structure" /></td>
<td>0.819</td>
<td>165.3</td>
</tr>
<tr>
<td>1911-F21</td>
<td><img src="image" alt="Structure" /></td>
<td>0.5505</td>
<td>158.2</td>
</tr>
<tr>
<td>1599-F21</td>
<td><img src="image" alt="Structure" /></td>
<td>0.5965</td>
<td>157.8</td>
</tr>
<tr>
<td>1503-I21</td>
<td><img src="image" alt="Structure" /></td>
<td>0.5465</td>
<td>155.7</td>
</tr>
<tr>
<td>1911-M20</td>
<td><img src="image" alt="Structure" /></td>
<td>0.5295</td>
<td>151.2</td>
</tr>
</tbody>
</table>
**Figure 17: Heatmaps of the cherry-pick screening plates.** Colors represent optical density, with dark red reflecting higher growth. In cherry-picking experiments against PDE11, compounds with OD > 0.179 were defined as strong, 0.167-0.179 as moderate, and 0.15-0.167 were defined as weak PDE11 inhibitors. Only moderate and strong PDE11 hits were taken into further consideration. In the screen against PDE10, compounds with OD > 0.23 were defined as strong, 0.2-0.23 were defined as moderate, 0.195-0.2 were defined as weak PDE10 hits. In the screen against PDE5, compounds with OD > 0.25 were defined as strong, 0.22-0.25 were defined as moderate, 0.2-0.22 were defined as weak PDE5 hits.
Figure 17

- Plate 1: 126 PDE11 hits
- Plate 2: 25 PDE10 hits
- Plate 3: 15 PDE5 hits

Legend:
- Strong hit
- Moderate hit
- Weak hit
Table 8: PDE5 and/or PDE10 hits in cherry-picking experiments.

Only the strong PDE11/5 and PDE11/10 inhibitors are presented for dual inhibitors.

<table>
<thead>
<tr>
<th>ICCB plate/well #</th>
<th>Structure</th>
<th>PDE11 primary screen Ave Abs</th>
<th>PDE11 primary screen Comp Z</th>
<th>PDE11 cherry-picking OD</th>
<th>PDE10 cherry-picking OD</th>
<th>PDE5 cherry-picking OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE11/10/5 inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1716-A7</td>
<td><img src="image1" alt="Structure" /></td>
<td>0.46</td>
<td>36</td>
<td>0.253</td>
<td>0.248</td>
<td>0.207</td>
</tr>
<tr>
<td>2091-H15</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.294</td>
<td>47.8</td>
<td>0.449</td>
<td>0.328</td>
<td>0.307</td>
</tr>
<tr>
<td>Strong PDE11/5 inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1808-O6</td>
<td><img src="image3" alt="Structure" /></td>
<td>0.45</td>
<td>66.1</td>
<td>0.328</td>
<td>0.133</td>
<td>0.919</td>
</tr>
<tr>
<td>1804-N8</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.43</td>
<td>71.7</td>
<td>0.314</td>
<td>0.14</td>
<td>0.769</td>
</tr>
<tr>
<td>1808-M6</td>
<td><img src="image5" alt="Structure" /></td>
<td>0.43</td>
<td>62.4</td>
<td>0.289</td>
<td>0.124</td>
<td>0.393</td>
</tr>
<tr>
<td></td>
<td>Structure</td>
<td>IC50 (pM)</td>
<td>IC50 (pM)</td>
<td>IC50 (pM)</td>
<td>IC50 (pM)</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>1610-E1</td>
<td><img src="image1.png" alt="Image" /></td>
<td>0.15</td>
<td>29.6</td>
<td>0.225</td>
<td>0.189</td>
<td>0.338</td>
</tr>
<tr>
<td>1842-D11</td>
<td><img src="image2.png" alt="Image" /></td>
<td>0.38</td>
<td>70.3</td>
<td>0.323</td>
<td>0.137</td>
<td>0.273</td>
</tr>
<tr>
<td>1889-H3</td>
<td><img src="image3.png" alt="Image" /></td>
<td>0.193</td>
<td>39.4</td>
<td>0.177</td>
<td>0.16</td>
<td>0.272</td>
</tr>
<tr>
<td><strong>Strong PDE11/10 inhibitors</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1911-C16</td>
<td><img src="image4.png" alt="Image" /></td>
<td>0.29</td>
<td>70</td>
<td>0.265</td>
<td>0.291</td>
<td>0.198</td>
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<tr>
<td><strong>PDE10 inhibitors</strong></td>
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<tr>
<td>1481-O22</td>
<td><img src="image5.png" alt="Image" /></td>
<td>0.41</td>
<td>33.2</td>
<td>0.159</td>
<td>0.209</td>
<td>0.162</td>
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<tr>
<td>1534-F17</td>
<td><img src="image6.png" alt="Image" /></td>
<td>0.21</td>
<td>22.3</td>
<td>0.177</td>
<td>0.208</td>
<td>0.16</td>
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<tr>
<td>527-P6</td>
<td><img src="image1.png" alt="Molecule" /></td>
<td>0.68</td>
<td>26.1</td>
<td>0.113</td>
<td>0.208</td>
<td>0.154</td>
</tr>
<tr>
<td>1802-D11</td>
<td><img src="image2.png" alt="Molecule" /></td>
<td>0.22</td>
<td>26.7</td>
<td>0.141</td>
<td>0.205</td>
<td>0.161</td>
</tr>
<tr>
<td>1542-H20</td>
<td><img src="image3.png" alt="Molecule" /></td>
<td>0.2</td>
<td>22</td>
<td>0.199</td>
<td>0.2</td>
<td>0.135</td>
</tr>
<tr>
<td>618-E3</td>
<td><img src="image4.png" alt="Molecule" /></td>
<td>0.28</td>
<td>29.2</td>
<td>0.16</td>
<td>0.2</td>
<td>0.128</td>
</tr>
<tr>
<td><strong>PDE5 inhibitors</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1647-B8</td>
<td><img src="image5.png" alt="Molecule" /></td>
<td>0.22</td>
<td>20.9</td>
<td>0.181</td>
<td>0.157</td>
<td>0.439</td>
</tr>
<tr>
<td>1864-B10</td>
<td><img src="image6.png" alt="Molecule" /></td>
<td>0.18</td>
<td>26.6</td>
<td>0.12</td>
<td>0.161</td>
<td>0.21</td>
</tr>
<tr>
<td>1488-C6</td>
<td><img src="image7.png" alt="Molecule" /></td>
<td>0.56</td>
<td>48</td>
<td>0.102</td>
<td>0.162</td>
<td>0.204</td>
</tr>
</tbody>
</table>
Figure 18: Summary of cherry-pick screen results for validation and counter-selection of primary screen hits against PDE11, PDE5, and PDE10. (A) Optical densities of PDE5-expressing strain (left panel) and PDE10-expressing strain (right panel) are plotted against those of the PDE11-expressing strain. The colors represent Composite Z scores against PDE11 in cherry-picking experiments. (B) 99 of the 126 confirmed PDE11 hits were specific for PDE11 in cherry-picking experiments.
Figure 18

A

B
3.4.3. Selection of compounds for secondary assays

Compounds that were PDE11-specific in the cherry-picking experiments were analyzed to select a group of high-priority candidates for secondary assays. In this process, compounds that produced higher OD and Composite Z scores in both primary and cherry-picking screens were prioritized. Molecules that were smaller than 250 g/mole, or larger than 450g/mole, or had steroid-like structures were avoided for preventing possible membrane-penetration problems or off-target effects in mammalian cells. Other structural properties that make compounds suitable candidates for medicinal chemistry to improve their pharmacokinetic properties were also considered, such as selecting ones with <4 interlocking rings. In cases when several structurally-related compounds appeared as hits (such as the multiple hits in the 1911 plate), the most effective one or two representative compounds that shared the common structural core were selected for further analysis. This approach led to the identification of 22 lead candidates for further testing in secondary assays.

It was surprising that many compounds identified in the primary screen were not validated as PDE11 hits in the cherry-picking experiments. One possible explanation for this could be the different delivery method in the cherry-pick screens, as pocket tips were used in these experiments, rather than the steel pin arrays in the primary screens. To test whether the pocket tip-delivery method created a problem in introducing compounds to microtiter dishes, I performed a 5FOA growth assay with 40 compounds
that appeared as strong hits in the initial screen, but performed poorly in the cherry-pick experiments. Half of these compound promoted growth of the PDE11-expressing strain to >0.5 OD at 25 µM (Figure 19), which suggests that the delivery of compounds into microtiter dishes using pocket tips fails to allow some compounds to dissolve in the growth medium, and is at least partially responsible for the low number of validated hits in the cherry-picking screens. 5 compounds, which produced high ODs and Composite Z scores in the primary screen and resulted in >0.6 OD at 25 µM in this experiment, were included in the list of candidates for secondary assays.

After screening the first ~30,000 compounds in the primary screen (prior to the cherry-picking experiments), 8 strong hits (BC11-1 to BC11-8) were acquired to test for detection of PDE11 inhibitors in secondary assays. These compounds, except for BC11-5 which was included in the cherry-picking experiments, were tested in secondary assays before the primary screens were completed.

In addition to the PDE11-selective candidates, two compounds that appeared as PDE5/11 inhibitors (1804-N8 and 1842-D11), and one that appeared as a PDE10/11 inhibitor (1911-C16) were also purchased to use as controls for PDE5 and PDE10 inhibition in secondary assays. This resulted in a list of 38 candidate compounds for further testing in secondary assays for identifying PDE11-selective inhibitors (Table 9).
Figure 19: 5FOA growth assays with compounds that produced high ODs in the primary screen but performed poorly in cherry-picking experiments. 40 compounds that performed poorly in cherry-picking experiments were tested at upto 70 µM in 5FOA growth assays against the PDE11-expressing strain, by delivery from the stock cherry-picking plates. 5 compounds that produced OD >0.6 at 25 µM (1865-A01, 1786-A16, 1479-I02, 1451-C14, and 1746-G05) were obtained for further testing in secondary assays. BC76 was used as a positive control.
Figure 19

![Graph showing Optical Density vs. Compound concentration (µM)]
<table>
<thead>
<tr>
<th>Comp ID</th>
<th>Structure</th>
<th>ICCB Plate-well #</th>
<th>PDE11 Comp Z</th>
<th>PDE11 1° screen Ave OD</th>
<th>PDE11 cherry-picking OD</th>
<th>PDE5 cherry-picking OD</th>
<th>PDE10 cherry-picking OD</th>
<th>Reason for selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC11 -1</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>1597-A7</td>
<td>196.1</td>
<td>0.723</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -2</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>1602-H14</td>
<td>117.5</td>
<td>0.463</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -3</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>1478-J21</td>
<td>33.5</td>
<td>0.412</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -4</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>1477-A15</td>
<td>75.6</td>
<td>0.838</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -5</td>
<td>1478-A7 (Later renamed as BC11-35)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC11 -6</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>1488-C6</td>
<td>48.1</td>
<td>0.561</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -7</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>1492-F9</td>
<td>43.5</td>
<td>0.514</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2° assays</td>
</tr>
<tr>
<td>BC11 -8</td>
<td>1483-D10</td>
<td>36</td>
<td>0.437</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Initial pick to optimize 2(^\circ) assays</td>
<td></td>
</tr>
<tr>
<td>-------</td>
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<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>BC11 -9</td>
<td>1548-A5</td>
<td>85.7</td>
<td>0.558</td>
<td>0.307</td>
<td>0.183</td>
<td>0.152</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td>BC11 -10</td>
<td>1595-J3</td>
<td>76.5</td>
<td>0.328</td>
<td>0.295</td>
<td>0.200</td>
<td>0.162</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td>BC11 -11</td>
<td>587-L11</td>
<td>75.6</td>
<td>0.564</td>
<td>0.36</td>
<td>0.16</td>
<td>0.141</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td>BC11 -12</td>
<td>1395-H20</td>
<td>52.8</td>
<td>0.321</td>
<td>0.228</td>
<td>0.156</td>
<td>0.146</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td>BC11 -13</td>
<td>1746-G5</td>
<td>39.3</td>
<td>0.444</td>
<td>0.216</td>
<td>0.135</td>
<td>0.143</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td>BC11 -14</td>
<td>1396-A15</td>
<td>66.3</td>
<td>0.381</td>
<td>0.202</td>
<td>0.123</td>
<td>0.154</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td><strong>BC11-15</strong></td>
<td>1786-A16</td>
<td>82.1</td>
<td>0.451</td>
<td>0.158</td>
<td>0.162</td>
<td>0.156</td>
<td>High OD in primary screen, confirmed in 5FOA growth assay</td>
<td></td>
</tr>
<tr>
<td><strong>BC11-16</strong></td>
<td>1724-J17</td>
<td>44.1</td>
<td>0.54</td>
<td>0.265</td>
<td>0.164</td>
<td>0.142</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td><strong>BC11-17</strong></td>
<td>1686-J3</td>
<td>76.2</td>
<td>0.259</td>
<td>0.273</td>
<td>0.146</td>
<td>0.164</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
</tr>
<tr>
<td><strong>BC11-18</strong></td>
<td>1706-G5</td>
<td>47.4</td>
<td>0.553</td>
<td>0.254</td>
<td>0.162</td>
<td>0.132</td>
<td>Strong hit confirmed in cherry-picking</td>
<td></td>
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<tr>
<td><strong>BC11-19</strong></td>
<td>1522-A21</td>
<td>107.4</td>
<td>0.444</td>
<td>0.202</td>
<td>0.185</td>
<td>0.138</td>
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<td><strong>BC11-20</strong></td>
<td>1442-A13</td>
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<td>0.739</td>
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<td><strong>BC11-21</strong></td>
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<td>80.2</td>
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<td>1835-G21</td>
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<td>0.146</td>
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<td>0.200</td>
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<td>Compound ID</td>
<td>IC50</td>
<td>Selectivity</td>
<td>Purity</td>
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<td>High OD in primary screen, confirmed in 5FOA growth assay</td>
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</tbody>
</table>
3.5. SECONDARY ASSAYS TO IDENTIFY PDE11-SPECIFIC INHIBITORS

I used the following approach to identify PDE11-selective inhibitors: the 38 lead compounds were characterized by 5FOA growth assays using yeast strains that express PDEs representing 10 of the 11 mammalian PDE families (with the exception of PDE6) (Table 4). Compounds that promoted growth of only the PDE11-expressing strain in the yeast growth assays were further tested using *in vitro* enzyme assays against PDEs 5, 6, 10 (the three structurally most similar PDEs to PDE11), and PDE11, at 2 and 10 µM. Compounds that had IC\textsubscript{50} values <2 µM for PDE11, and >10 µM against PDEs 5, 6, and 10 in these experiments were classified as PDE11-specific candidates, and were further analyzed using *in vitro* enzyme assays against all 11 PDE families to confirm their selectivity and determine their precise IC\textsubscript{50} values.

6 compounds (BC11-11, BC11-15, BC11-19, BC11-20, BC11-28, BC11-38) were found to be PDE11-specific in 5FOA growth assays (Figure 20). BC11-20 failed to inhibit PDE11, PDE5, PDE6, or PDE10 *in vitro* (Figure 21). Since BC11-11 appeared to have an IC\textsubscript{50} value >2 µM against PDE11, it was also excluded from further consideration. BC11-15, BC11-19, BC11-28, and BC11-38 were potent inhibitors of PDE11 and did not inhibit PDEs 5, 6, and 10 in these experiments. These compounds were defined as PDE11-specific candidates and were further analyzed using *in vitro* enzyme assays against PDEs representing all 11 PDE families (PDE1-11).
Figure 20: 5FOA growth assays for identification of PDE11-specific inhibitors. 6 compounds promoted growth of only the PDE11-expressing strain. Compounds were tested up to 100 μM. Dose-response growth curves in the presence of ≤30 μM compound from one representative (with duplicate wells for each data point) of triplicate experiments are presented, to allow observation of responses to lower compound concentrations.
Figure 20


- BC11-11
- BC11-15
- BC11-19
- BC11-20
- BC11-28
- BC11-38

Graphs show optical density against concentration for different samples, with lines representing different PDE enzymes (PDE1 to PDE11).
Figure 21: *In vitro* enzyme assays for identification of PDE11-specific inhibitors.

Compounds that were specific for PDE11 in 5FOA growth assays were profiled against PDEs 5, 6, 10, and 11 using *in vitro* enzyme assays at 2 and 10 µM concentration. Graph represents % activity of enzyme compared to DMSO control.
Figure 21
Among the 8 compounds that were obtained prior to cherry-picking, 2 compounds (BC11-4 and BC11-8) were specific for promoting growth of the PDE11- and PDE10-expressing strains in 5FOA growth assays, and inhibited PDE11 in in vitro enzyme assays with \(IC_{50}\) values of 150 and 50 nM, respectively (Figure 22A). These two compounds also inhibited PDE10 in vitro, with \(IC_{50}\) values of 2 µM and 200 nM.

Several dual-specific candidates were also identified in the secondary assays. For example, BC11-21 and BC11-27 were specific for inhibiting PDE5 and PDE11 in in vitro enzyme assays (Figure 23A). In addition, two weak PDE11 inhibitors, BC11-12 and BC11-13 (\(IC_{50}\)s 2-10 µM), inhibit PDE10 with similar potency (Figure 23B). Although these compounds were excluded from further examination in this study aimed at identifying PDE11-selective inhibitors, they might serve as useful research tools as partially-selective PDE inhibitors in future studies.

The results of all secondary assays are summarized in Table 10. Overall, 4 compounds (BC11-15, BC11-19, BC11-28, BC11-38) were identified as PDE11-selective candidates in the secondary assays (Figure 24A). In order to confirm their potency and determine their \(IC_{50}\) values, BC11-15, BC11-19, BC11-28, and BC11-38 were evaluated using in vitro enzyme assays for their ability to inhibit PDEs representing all of the 11 PDE families (PDEs 1-11). These compounds display \(IC_{50}\) values \(\leq 330\) nM and >100-fold selectivity for PDE11 relative to other PDEs, with the exception of BC11-19 that is only
30-fold selective for PDE11 relative to PDE1 (Figure 24B). To my knowledge, these compounds are the first identified PDE11-selective inhibitors.

3.6. DETERMINING THE MECHANISM OF INHIBITION BY PDE11-SPECIFIC INHIBITORS

In order to identify the mechanism of inhibition by BC11-15, BC11-19, BC11-28, and BC11-38, in vitro enzyme assays with PDE11 using varying cGMP concentrations were performed. Lineweaver-Burk plots of 1/V vs. 1/[S] for each of the compounds were analyzed to determine their mechanism of inhibition.

The double reciprocal plots suggest that BC11-15, BC11-19, BC11-28, and BC11-38 increase the $K_m$ of the reactions without affecting the $V_{max}$ (Figure 25). These results suggest that all of these four compounds act as competitive inhibitors of PDE11.
Figure 22: 5FOA growth and *in vitro* enzyme assays using BC11-1 to BC11-8 compounds identify two PDE10/11 inhibitors. (A) BC11-4 and BC11-8 are selective for PDE10- and PDE11-expressing strains in 5FOA growth assays. (B) IC₅₀ values based on *in vitro* enzyme assays against PDE10 and PDE11 enzymes.
Figure 22

A

BC11-4

BC11-8

Optical Density

Optical Density

[BC11-4] (µM)

[BC11-8] (µM)

B

<table>
<thead>
<tr>
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<th>IC_{50} (µM)</th>
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</thead>
<tbody>
<tr>
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<td>PDE11</td>
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<tr>
<td>BC11-4</td>
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<tr>
<td>BC11-8</td>
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Figure 23: Dual-specific PDE5/11 and PDE10/11 inhibitor candidates identified in secondary assays. (A) BC11-21 and BC11-27 inhibit PDE5 and PDE11, but not PDE10. (B) BC11-12 and BC11-13 inhibit PDE10 and PDE11 with nearly equal potency in *in vitro* enzyme assays.
Figure 23

A

% activity

BC11-21 2μM

BC11-21 10μM

BC11-27 2μM

BC11-27 10μM

PDE11

PDE5

PDE10

B

% activity

BC11-12 2μM

BC11-12 10μM

BC11-13 2μM

BC11-13 10μM

PDE11

PDE10
### Table 10. Summary of secondary assay results

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Results in secondary assays</th>
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</thead>
<tbody>
<tr>
<td>BC11-1</td>
<td>Hits PDEs 1, 8, 10 in 5FOA growth assays</td>
</tr>
<tr>
<td>BC11-2</td>
<td>Hits PDEs 1 and 10 in 5FOA growth assays</td>
</tr>
<tr>
<td>BC11-3</td>
<td>Poor PDE11 inhibitor <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-4</td>
<td>Hits PDE10 in 5FOA growth and <em>in vitro</em> assays</td>
</tr>
<tr>
<td>BC11-6</td>
<td>Does not inhibit PDE11 <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-7</td>
<td>Poor PDE11 inhibitor <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-8</td>
<td>Hits PDE10 in 5FOA growth and <em>in vitro</em> assays</td>
</tr>
<tr>
<td>BC11-9</td>
<td>Poor PDE11 inhibitor <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-10</td>
<td>Hits PDE10 <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-11</td>
<td>Poor PDE11 inhibitor <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-12</td>
<td>Hits PDE10 <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-13</td>
<td>Hits PDE10 <em>in vitro</em></td>
</tr>
<tr>
<td>BC11-14</td>
<td>Does not inhibit PDE11 <em>in vitro</em></td>
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<tr>
<td>BC11-15</td>
<td>Selective and potent PDE11 inhibitor</td>
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<tr>
<td>BC11-16</td>
<td>Hits PDE10 <em>in vitro</em></td>
</tr>
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<td>BC11-17</td>
<td>Hits PDE10 in 5FOA growth assays</td>
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<tr>
<td>BC11-18</td>
<td>Hits PDE5 in 5FOA growth assays</td>
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<td>BC11-19</td>
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<td>BC11-21</td>
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<td>BC11-27</td>
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<td>BC11-28</td>
<td>Selective and potent PDE11 inhibitor</td>
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<tr>
<td><strong>BC11-29</strong></td>
<td>Does not inhibit PDE11 <em>in vitro</em></td>
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<td><strong>BC11-30</strong></td>
<td>Hits PDE10 <em>in vitro</em></td>
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<td>Selective and potent PDE11 inhibitor</td>
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<td><strong>BC11-39</strong></td>
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Figure 24: Four compounds identified as PDE11-selective inhibitors. (A) Summary of the screening process that led to the identification of four potent and selective PDE11-inhibitors. cAMP was included in the common hits with PDE8 screen. (B) Structures and IC$_{50}$ values of PDE11-specific inhibitors in in vitro enzyme assays. Values represent averages of at least three independent experiments. MW, molecular weight.
Figure 24

A

Primary screen of 198,382 compounds

1143 hits

Weak PDE11 hits

PDE8 hits

PDE4/7 hits

Counter screen of top 595 hits

Secondary assays (38)

4 PDE11-specific inhibitors

B

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<tr>
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Figure 25: BC11-15, BC11-19, BC11-28, and BC11-38 act as competitive inhibitors of PDE11. Lineweaver-Burk plots represent $1/V$ (min$\times$µg/µM) vs. $1/[cGMP]$ (µM$^{-1}$) for PDE11 activity for varying concentrations of cGMP, in the absence or presence of each of the PDE11-selective inhibitors. Velocity of reactions were calculated by multiplying the fraction of cGMP converted to 5’GMP with the substrate concentration, and dividing that value by the time of reaction. This number was then divided by the amount of enzyme to determine the rate of reaction per µg of enzyme. The graphs for compounds have the same y-axis intercept as the uninhibited enzyme, indicating that the $V_{max}$ of the reactions are unaffected while the $K_m$ increases, suggesting that these compounds act as competitive inhibitors of PDE11.
Figure 25

BC11-15

BC11-19

BC11-28

BC11-38

1/[cGMP] (μM⁻¹)

1/[cGMP] (μM⁻¹)
CHAPTER FOUR

DEMONSTRATING A BIOLOGICAL ROLE FOR PDE11 USING PDE11-SPECIFIC INHIBITORS - THE ROLE OF PDE11 IN CORTISOL PRODUCTION BY ADRENOCORTICAL CELLS

4.1. PDE11-SPECIFIC INHIBITORS ELEVATE cAMP LEVELS AND CORTISOL PRODUCTION IN ADRENOCORTICAL CELLS

PDE11 is expressed in the adrenal glands (D'Andrea et al., 2005), and PDE11A inactivating mutations were suggested as predisposing factors in the development of adrenocortical hyperplasia (Boikos et al., 2008; Carney et al., 2010; Horvath et al., 2006a; Horvath et al., 2006b). In these tumors, elevated cAMP levels and increased CREB phosphorylation result in excess cortisol release, leading to Cushing syndrome (Horvath et al., 2006a). In order to test whether our PDE11-specific inhibitors could be used to study biological roles of PDE11 in mammalian cells, I tested whether the phenotypes associated with PDE11 inactivation in Cushing syndrome (i.e., elevated cAMP and cortisol levels) could be mimicked by treatment of adrenocortical cells with these compounds. For this purpose, H295R human adrenocortical carcinoma cells, a well-described model for adrenal cell function and steroidogenesis studies (Rainey et
al., 1994), were treated with the PDE11-selective inhibitors, and their effects on cAMP and cortisol levels were analyzed.

H295R cells have the ability to produce all the adrenocortical steroids (i.e., mineralocorticoids, glucocorticoids, adrenal androgens) (Rainey et al., 1993). Cortisol production by adrenocortical cells is regulated via cAMP signaling pathway (Stratakis, 2009). Transcription of genes encoding several key enzymes involved in cortisol production by adrenocortical cells is activated by the cAMP-response element (CRE)-binding proteins, which include CREB, CREM, and ATF1. Rainey et al. has shown that the treatment of H295R cells with forskolin (an adenylate cyclase activator) causes a significant increase in cAMP production, which is further elevated by the addition of IBMX (Rainey et al., 1993). To test whether PDE11-specific inhibitors would have an effect on cAMP levels, H295R cells were treated with these compounds both in the absence and presence of forskolin. The nonselective PDE inhibitor IBMX was used as a control to determine the effect of inhibiting all PDE activity, with the exception of PDE8 and PDE9, in order to assess the relative importance of PDE11 on cAMP hydrolysis in these cells. Consistent with the data by Rainey et al. (1993), forskolin elevates cAMP levels as compared to a DMSO control, which is further increased several-fold by IBMX (Figure 26A). BC11-38 significantly elevates cAMP levels in H295R cells both in the absence and presence of forskolin, suggesting that PDE11 represents a significant proportion of the cAMP PDE activity in these cells.
Rainey et al. (1993) have also shown that forskolin treatment significantly elevates cortisol production by H295R cells. In order to test whether PDE11-specific inhibitors would cause a further elevation in cortisol production by adrenocortical cells, cortisol release by H295R cells was measured following a treatment with these compounds in the presence of forskolin. BC11-38 causes a significant increase in cortisol release by H295R cells, suggesting that PDE11 has a major role in regulating cortisol production in adrenocortical cells (Figure 26B).

The PDE11-specific inhibitors were used at 20 µM, the maximal effective concentration determined in these assays. IBMX was used at 500 µM to measure the effect of near complete inhibition of all PDEs with the exception of PDE8 and PDE9, since the IC<sub>50</sub> values for IBMX against various PDEs range from 2 to 50 µM. However, at this concentration, off-target effects of IBMX treatment may have prevented cells from increasing cortisol production.

These results demonstrate that BC11-38 is able to enter the cells and suggest that it increases cAMP and cortisol levels by inhibiting PDE11. The failure to elevate cAMP and cortisol levels by BC11-15, BC11-19, and BC11-28 suggests that these compounds either fail to enter the cells due to poor solubility in tissue culture media or have a detrimental effect on cells. Consistent with the latter idea, compounds BC11-15 and
BC11-28 have high LogP values (>5.5), suggesting that they may be too lipophilic for these assays. Alternatively, the effect of BC11-38 could be due to an off-target activity.

4.2. DEMONSTRATING THAT THE BIOLOGICAL EFFECTS OF BC11-38 ARE DUE TO PDE11 INHIBITION

4.2.1. Biological effects of PDE11-specific inhibitors on cell lines with different levels of PDE11 expression

To address the possibility that BC11-38 elevates cAMP levels and cortisol release by H295R cells in a PDE11-independent manner, the correspondance between PDE11 expression levels and the biological effects of PDE11-specific inhibitors was analyzed. For this purpose, relative PDE11 expression levels in six different cell lines were determined by RT-PCR. H295 has several-fold higher PDE11 expression compared to HeLa, Jurkat, T47D, DU145, and MDA-MB-231 cell lines (Figure 27).

The effects of the PDE11-specific inhibitors on cAMP levels and CREB phosphorylation in MDA-MB-231 cells, which express lower levels of PDE11, were evaluated. Neither BC11-38 nor the other three PDE11-specific inhibitors increase cAMP levels or CREB phosphorylation in MDA-MB-231 cells (Figure 28). These results support the hypothesis that the biological effects by BC11-38 in adrenocortical cells are due to PDE11 inhibition.
Figure 26: PDE11-selective inhibitors elevate cAMP levels and cortisol production in H295R adrenocortical cells. (A) cAMP levels of H295R cells following a 2 h treatment with PDE11-specific inhibitors (20 µM) in the absence or presence of 10 µM forskolin. The nonselective PDE inhibitor IBMX was used at 500 µM as a positive control. (B) Cortisol release by H295R cells following a 24 h treatment with PDE11-specific inhibitors (20 µM) or IBMX (500 µM) in the presence of 10 µM forskolin. Data are presented as % of forskolin+DMSO treated cells. Values represent the averages of two or more independent experiments for each assay performed in duplicate ±S.E.M. (*p<0.05, **p<0.01; as determine by one-way ANOVA).
Figure 26

A  
Figure 26A shows the cAMP levels in different conditions. The graph displays the cAMP levels (pmol/mg protein) for various treatments. The x-axis represents different treatments (DMSO, BC11-15, BC11-19, BC11-28, IBMX, BC11-15 + Forskolin, BC11-19 + Forskolin, BC11-28 + Forskolin, BC11-38 + Forskolin) and the y-axis represents the cAMP levels (pmol/mg protein). The bars with asterisks indicate significant differences compared to the control group.

B  
Figure 26B illustrates the cortisol production as a percentage of DMSO (%DMSO). The x-axis represents different treatments (DMSO, BC11-15, BC11-19, BC11-28, BC11-38, IBMX) and the y-axis represents cortisol production (%DMSO). The bars with asterisks indicate significant differences compared to the control group.
Figure 27: H295R cells express higher levels of PDE11 expression compared to HeLa, Jurkat, T47D, DU145, and MDA-MB-231 cells. Semi-quantitative RT-PCR for PDE11 mRNA was performed in various cell lines. PDE11 expression was normalized to the expression level of the RPLP0 reference gene.
Figure 27

Relative PDE11 Expression

HeLa  Jurkat  T47D  DU145  MDA-MB-231  H295R
Figure 28: BC11-38 and the other PDE11-specific inhibitors do not increase cAMP levels or CREB phosphorylation in MDA-MB-231 cells. (A) cAMP levels and (B) CREB phosphorylation in MDA-MB-231 cells following a 2 h treatment with BC11-38 and derivatives (20 µM) in the presence of 10 µM forskolin. Cells were lysed and proteins were subjected to immunoblotting with a p-CREB/ATF-1 antibody. Values represent the averages of three separate experiments for each assay performed in duplicate ±S.E.M. (**p<0.01; determined by one-way ANOVA).
Figure 28

A  MDA-MB-231

![Bar graph showing cAMP levels in different treatments.](image)

B  MDA-MB-231

![Western blot images showing P-CREB and mTOR levels.](image)
4.2.2. The effects of BC11-38 structural derivatives on PDE inhibition and on cAMP/cortisol levels and ATF-1 phosphorylation in H295R adrenocortical cells

To further test whether the biological activities of BC11-38 in adrenocortical cells is due to PDE11 inhibition, four structural derivatives of BC11-38 that differ by a single methyl or methoxy group were acquired. The potency of these compounds for inhibiting each of the 11 PDE families (PDE1-11) were determined using in vitro enzyme assays (Figure 29). The potency of these compounds for PDE11 inhibition is BC11-38-1 > BC11-38-2 > BC11-38-3 > BC11-38-4.

The ability of the BC11-38 structural derivatives to elevate cAMP levels and cortisol production in H295R cells are consonant with their potency for PDE11 inhibition (BC11-38-1 > BC11-38-2 > BC11-38-3 > BC11-38-4) (Figure 30A and 30C). Since transcription of genes encoding several key enzymes involved in cortisol production by adrenocortical cells is activated by the cAMP-response element (CRE)-binding proteins, and ATF-1 and CREM are the major proteins that bind the CRE in H295R cells, which lack CREB, and are phosphorylated by PKA in response to elevated cAMP levels to activate transcription, the phosphorylation state of ATF-1 in H295R cells was analyzed (Groussin et al., 2000; Rosenberg et al., 2002b; Wang et al., 2000). BC11-38 and its derivatives elevate ATF-1 phosphorylation in a manner that corresponds with their potency against PDE11 (Figure 30B).
Figure 29: Four structural derivatives of BC11-38. Structures and IC$_{50}$ values of PDE11-specific inhibitors in *in vitro* enzyme assays are presented. Values represent averages of at least three independent experiments. MW, molecular weight.
**Figure 29**

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Figure 30: Biological activities of BC11-38 and derivatives are consonant with their potency for PDE11 inhibition. (A) cAMP levels and (B) ATF-1 phosphorylation in H295R adrenocortical cells following a 2 h treatment with BC11-38 and derivatives (20 μM) in the presence of 10 μM forskolin. Cells were lysed and proteins were subjected to immunoblotting with a p-CREB/ATF-1 antibody. (C) Cortisol release by H295R cells following a 24 h treatment with BC11-38 and derivatives (20 μM) or IBMX (500 μM) in the presence of 10 μM forskolin. Data are presented as % of forskolin+DMSO treated cells. Values represent the averages of three separate experiments for each assay performed in duplicate ±S.E.M. (*p<0.05, **p<0.01; determined by one-way ANOVA).
Figure 30

A

B

C

CAMP (pmol/mg protein)

DMSO

BC11-38

BC11-38-1

BC11-38-2

BC11-38-3

BC11-38-4

IBMX

+Forskolin

p-ATF1

AKT

+Forskolin

Cortisol (% DMSO)

DMSO

BC11-38

BC11-38-1

BC11-38-2

BC11-38-3

BC11-38-4

IBMX
4.2.3. The effects of BC11-38 structural derivatives on cAMP levels and ATF-1/CREB phosphorylation in HeLa cells

Biological effects of BC11-38 derivatives were also analyzed in HeLa cells that have very low levels of PDE11 expression. Consistent with PDE11 inhibition as the primary effect of these compounds, neither BC11-38 nor its derivatives affect cAMP levels or CREB (and ATF-1) phosphorylation levels in HeLa cells (Figure 31).

Taken together, these results suggest that the biological effects of BC11-38 and related compounds in adrenocortical cells are due to PDE11 inhibition. BC11-38, along with two derivatives, elevates cAMP levels and cortisol production in adrenocortical cells in a PDE11-specific manner, mimicking the phenotypes observed in Cushing syndrome. The results with the adrenocortical cells demonstrate that these compounds could serve immediately as useful research tools to study the biological roles of PDE11 in mammalian cells.
Figure 31: BC11-38 and related compounds do not increase cAMP levels or CREB phosphorylation in HeLa cells. (A) cAMP levels and (B) ATF-1 phosphorylation in HeLa cells following a 2 h treatment with BC11-38 and derivatives (20 µM) in the presence of 10 µM forskolin. Cells were lysed and proteins were subjected to immunoblotting with a p-CREB/ATF-1 antibody. Values represent the averages of three separate experiments for each assay performed in duplicate ±S.E.M. (**p<0.01; determined by one-way ANOVA).
Figure 31

A

B

+ Forskolin

p-CREB
p-ATF1
CREB

+ Forskolin
4.3. INVESTIGATION OF BC11-19 AND BC11-28 STRUCTURAL DERIVATIVES TO IDENTIFY ADDITIONAL PDE11-SPECIFIC INHIBITORS WITH IMPROVED BIOLOGICAL ACTIVITY

In order to identify the pharmacophore for BC11-19 and BC11-28 that would have improved biological activity in mammalian cells while retaining PDE11-specificity, I analyzed five structural derivatives of BC11-19 and eleven derivatives of BC11-28 for their inhibitory profile on PDEs, and their ability to elevate cAMP levels in adrenocortical cells (Figure 32).

As an initial step, the compounds were tested at 0.2 and 2 µM for their ability to inhibit PDE11 in *in vitro* enzyme assays. Compounds that had IC$_{50}$ values <2 µM for PDE11 in these experiments were further tested against PDE5 and PDE10. None of the BC11-19 structural derivatives that were tested in these assays appeared to have IC$_{50}$ values <2 µM for PDE11. Four analogs of BC11-28 (BC11-28-7, BC11-28-8, BC11-28-10, BC11-28-11) were found to be highly potent inhibitors of PDE11, and were inactive against PDE5 and PDE10 (Figure 33A). These four compounds were analyzed for their ability to elevate cAMP levels in H295R adrenocortical cells. Similar to BC11-28, none of these structural analogs increased cAMP levels in H295R cells (Figure 33B). This might be explained by the poor solubility of these compounds in the growth media, as compound crystals were observed in the culture media when the compounds were added.
Figure 32: Structural analogs of BC11-19 and BC11-28. Five derivatives of BC11-19 and eleven derivatives of BC11-28 were obtained for identification of potential compounds with improved biological activity in mammalian cell culture studies, while retaining PDE11-specificity.
**Figure 32**

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**Figure 33:** The effects of BC11-19 and BC11-28 derivatives on PDE inhibition and cAMP levels in adrenocortical cells. (A) The structural derivatives of BC11-19 and BC11-28 that had IC$_{50}$ < 2 µM for PDE11 in initial profiling experiments were tested against PDE5 and PDE10 in *in vitro* enzyme assays. % enzyme activity compared to DMSO control are presented. (B) Four BC11-28 derivatives that were PDE11-selective in the *in vitro* assays were tested for their ability to elevate cAMP levels in H295R adrenocortical cells. cAMP levels in H295R cells following a 2 h treatment with the compounds (20 µM) in the presence of 10 µM forskolin are presented. IBMX was used at 500 µM.
Figure 33

A

![Bar chart showing % activity with different concentrations for PDE11, PDE5, and PDE10](chart)

B

![Bar chart showing cAMP (pmol/mg protein) levels with different treatments](chart)

+ Forskolin
CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTIONS

Phosphodiesterases are now recognized as key regulators of several physiological processes. They have proven to be valuable targets for therapeutic intervention in various diseases including erectile dysfunction, COPD, and pulmonary hypertension (Bender and Beavo, 2006; Francis et al., 2011). There has been remarkable progress in the understanding of the structure, regulation, and function of PDEs in the last few years, which has significantly benefitted from specific PDE inhibitors as research tools. These selective inhibitors also hold potential as therapeutic agents in the treatment of diseases that are associated with alterations in cyclic nucleotide signaling.

The functional importance of PDE11 has been suggested via genome-wide association studies (GWASs) that link it to a variety of cancers, psychiatric diseases, asthma, and Cushing syndrome. However, studies to examine its biological role in different tissues and these disease states have been hampered by the lack of selective pharmacological reagents. This study demonstrates the use of a fission yeast-based screen to identify potent and selective human PDE11 inhibitors. One inhibitor discovered in this screen, BC11-38, along with two derivatives, elevates cAMP levels and cortisol production in adrenocortical cells in a PDE11-specific manner, mimicking the phenotypes observed in
Cushing syndrome. These compounds can serve as useful research tools to study the biological roles of PDE11 in mammalian cells.

5.1. DEVELOPMENT AND PERFORMANCE OF A YEAST-BASED HTS FOR IDENTIFYING PDE11-SPECIFIC INHIBITORS

To address the need for PDE11-specific inhibitors, I developed and performed a yeast-based high throughput screen for PDE11 inhibitors. The screen employs genetically engineered yeast strains whose growth behavior reflects the activity of heterologously-expressed human PDE11. A high throughput screen of ~200,000 compounds was performed that identified 1143 PDE11 inhibitor candidates. 595 of these compounds (0.3% of all screened compounds) were retested in cherry-picking experiments against PDE11 for confirmation, and against PDE5 and PDE10 for elimination of nonselective hits. This led to the identification of 38 compounds that were further analyzed in secondary assays, which involved yeast growth assays with strains expressing 10 of the 11 PDE families and in vitro enzyme assays using enzymes from all 11 PDE families. These secondary assays allowed me to identify four highly potent and selective PDE11 inhibitors, which, to my knowledge, are the first identified PDE11-selective inhibitors.

The yeast-based screening platform has several features that aid in the identification of biologically-active PDE inhibitors. First, the target PDEs are full-length proteins expressed in eukaryotic cells, requiring inhibition to occur in the protein-dense yeast
cytosol, which resembles the human cytoplasmic environment. Second, compounds are detected by their ability to stimulate cell growth, so that the identified compounds must be cell-permeable, chemically-stable during the 48 h growth period, and non-toxic to S. pombe. This last feature is a proxy for high-specificity, as compounds that promiscuously bind proteins would likely retard or inhibit growth. Therefore, the compounds identified using this system already have certain characteristics that may make them attractive candidates for drug development. Other favorable features of this screen include the ability to detect both active-site and allosteric inhibitors and the use of a simple readout to rapidly screen large compound libraries. This 384-well format assay allowed screening of ~200,000 compounds, which is larger than any previously published yeast-based HTS, to our knowledge. The ability to identify potent PDE inhibitors that are biologically-active in mammalian cells in this and other HTSs confirms that S. pombe is well-suited for chemical screening. Using strains that express 10 of the 11 PDE families, I was able to profile inhibitor specificity and eliminate the majority of nonselective compounds prior to performing *in vitro* enzyme assays. Just as I used data from a PDE8 inhibitor screen to eliminate some compounds from further consideration, future PDE inhibitor screens will benefit from the database developed from this and previous screens to filter out nonselective PDE inhibitors and compounds that act in a PDE-independent manner.

This PDE11 inhibitor screen is the first application of our yeast-based platform to a HTS that identifies compounds based on their ability to inhibit cGMP hydrolysis. PDE11-
specific inhibitors discovered in this screen inhibit both cAMP- and cGMP-hydrolysis by PDE11 with similar IC_{50}s in in vitro enzyme assays. This study shows that the yeast-based screening platform, which was previously used in HTS of cAMP-specific PDEs 4, 7, and 8, can be used to develop screens for inhibitors of any PDE that display cAMP- or cGMP-hydrolyzing activity when expressed in fission yeast. Another potential use of this system could be to assess the effect of mutations in PDE genes on enzyme function, regulation, or sensitivity to pharmacological agents, and to characterize different PDE alleles. Expressing different alleles of PDE11A in this system, and comparing their activity or sensitivity to specific inhibitors would be an efficient way to elucidate the functional significance of various SNPs in PDE11A gene identified in GWASs. One could also perform genetic screens in yeast to detect mutations in PDE11A that alter enzyme sensitivity to specific inhibitors, which would indicate the sites that are important for inhibition by a particular compound. Finally, the yeast-based platform could also be used to discover PDE activators that stimulate Ura⁺ growth or reduce 5FOA^{R} growth in response to exogenous cAMP and cGMP. In an attempt to identify potential PDE11 activator candidates using the data from PDE11 inhibitor HTS, compounds were sorted based on their ability to reduce 5FOA^{R} growth compared to the negative controls. These results were compared with data from PDE8 inhibitor screen to eliminate compounds that were toxic to both strains. When these activator candidates were tested in 5FOA growth and in vitro enzyme assays, they did not activate PDE11. The lower absorbances they produced in the HTS were likely due to toxicity in yeast, and the differences between the ODs in PDE11 and PDE8 screens could be due to the intrinsic growth
differences of the two strains in their specific optimized screening conditions. It might be more likely to discover PDE11 activators in a screen that uses higher cGMP concentrations, by selecting compounds that reduce 5FOA^R growth while producing a higher growth rate than that of a “no cGMP” control. In the future, screens will need to be optimized to identify activator compounds based on stimulation of Ura^+ growth or reduction of 5FOA^R growth to facilitate the identification of authentic PDE11 activators.

In the HTS, candidate hit compounds were defined based on their Composite Z scores, which reflect both the level of growth stimulation and the reproducibility of the replicate assays. The Composite Z scores for the BC76 positive control were between 26 to 148. This wide range resulted from variations in the level of cell growth stimulation on different days of the experiment, most likely due to the temperature changes that the cells encountered at the screening facility or while being carried there from the laboratory. The temperature conditions affected cell growth in all wells, which was also reflected in variations among the absorbances and standard deviations of the negative controls. Hit compounds were defined based on comparison with the Composite Z scores of the positive controls (>26 were defined as moderate/strong hits). This definition prevented overlooking several hits that produced slightly lower absorbances. For example, one of the four highly potent PDE11-selective inhibitors, BC11-28, produced an average OD of 0.309 in the initial screen that might have prevented its identification as a hit if the classification of compounds had been based only on absorbance values. However, since the average negative control absorbance and the standard deviation
were 0.075 and 0.004, respectively in this assay, BC11-28 produced a Composite Z score of 77.8, and was identified as a strong hit in the initial screens.

One unexpected result was that many of the compounds identified in the initial screen were not validated as PDE11 hits in cherry-picking experiments. This is likely due to the different delivery method as the cherry-pick screens use pocket tips rather than the steel pin arrays used in the primary screens. When such compounds were retested in 5FOA growth assays, half of them were confirmed to promote growth of the PDE11-expressing strain. These results suggest that the pocket tip method of introducing compounds to microtiter dishes fails to allow some compounds to dissolve in the growth medium. The retesting step allowed identification of many potent PDE11 inhibitors that would have been missed based on the cherry-picking results, one of which is the selective inhibitor BC11-15.

It is noteworthy that the four most selective PDE11 inhibitors were not among the strongest hits in the primary and cherry-picking screens. BC11-19 was the 36th, BC11-38 was the 58th, BC11-15 was the 74th, and BC11-28 was the 83rd strongest hit in the HTS as defined by their Composite Z scores. The majority of the strongest hits in the primary screen that outperformed all positive controls were also hits in previous PDE4, 7, and 8 inhibitor screens by our lab (Table 7). These compounds could either be nonselective PDE inhibitors, or affect cell growth via PDE-independent mechanisms. BC11-1, which
was obtained and tested in secondary assays prior to the end of the initial screens, was the 4th strongest compound in the HTS, and was found to inhibit PDEs 1, 8, and 10 in 5FOA growth assays. The strongest hit that was acquired after completion of the initial and cherry-picking screens was BC11-30, which was the 12th strongest hit in the primary screens. This compound was found to inhibit PDE10 in vitro, thus was eliminated as a nonselective inhibitor. These results demonstrate the indispensability of secondary assays for identifying selective and potent PDE inhibitors.

Although the majority of the results of yeast growth assays were confirmed in in vitro enzyme assays, some compounds that were identified as PDE11 inhibitors in 5FOA growth assays were not found to inhibit PDE11 in vitro. The strongest and most selective of these compounds was BC11-20, which was highly potent and specific for the PDE11-expressing strain in 5FOA growth assays, but failed to inhibit PDE11 in in vitro enzyme assays. Since this compound was specific to promote growth of only the PDE11-expressing strain, it is unlikely that the growth response was due to a PDE-independent mechanism. It is possible that the lack of in vitro inhibition by BC11-20 could be due to the differences in the conditions of in vitro versus yeast growth assays, such as different substrate and ion concentrations. It is also possible that the intracellular environment where the enzyme is present in yeast might affect its inhibition by certain chemicals, or the pH of the 5FOA medium might influence compound structure. BC11-20 does not inhibit PDEs 5, 6, or 10 in in vitro enzyme assays, either. The lack of inhibition by this compound in in vitro assays would prevent the assessment of its specificity, and since a
number of compounds were already confirmed as PDE11-specific inhibitors using \textit{in vitro} enzyme assays as well, only the compounds that produced consistent results in the two types of assays were taken into further consideration.

The overall frequency of strong and moderate hits in this screen was 0.36\%, however this frequency varied significantly among the compound libraries. The highest frequencies of hits were observed in the Actimol TimTec 1 (0.8\%), Chembridge 3 (0.7\%), and ChemDiv 6 (0.64\%) libraries, which are among the most recently prepared libraries at ICCB. In contrast, the lowest frequency of hits was observed in the ChemDiv 1 library (0.04\%), which may reflect the fact that this was one of the oldest libraries screened and repeated freezing and thawing during previous screens may have adversely affected these compounds. Interestingly, the four compounds presented in Figure 24 came from four different libraries produced by four different companies (BC11-15, Enamine T0515-1965; BC11-19, Maybridge BTB 12009; BC11-28, ChemDiv K405-0344; BC11-38, Life Chemicals F0579-0060).

5.2. PDE11-SELECTIVE INHIBITORS ELEVATE cAMP LEVELS AND CORTISOL PRODUCTION IN ADRENOCORTICAL CELLS

\textit{PDE11A} inactivating mutations were suggested as predisposing factors in the development of adrenocortical hyperplasia (Horvath et al., 2006a). In these tumors, elevated cAMP levels and increased CREB phosphorylation result in excess cortisol
release, leading to Cushing syndrome. In this study, I confirm a role for PDE11 in cortisol production by adrenocortical cells using selective inhibitors. One of the four PDE11-specific inhibitors, BC11-38, along with two structural analogs, BC11-38-1 and BC11-38-2, elevates cAMP levels and cortisol production in human adrenocortical cells, mimicking the phenotypes associated with Cushing syndrome. These compounds are commercially-available reagents that are suitable for mammalian cell culture studies to evaluate PDE11 function. The results obtained in adrenocortical cells demonstrate that these inhibitors can serve as research tools even prior to medicinal chemistry approaches and as leads for the development of novel therapeutics for the treatment of adrenal insufficiencies.

The consonance between the potency of BC11-38 and its structural derivatives on PDE11 inhibition and their ability to elevate cAMP levels, ATF-1 phosphorylation, and cortisol levels in H295R cells suggests that the biological effects by these compounds are due to PDE11 inhibition. The lack of an effect on cAMP levels and CREB or ATF-1 phosphorylation by these compounds in cell lines with very low levels of PDE11 expression supports this hypothesis. Due to their biological activity, biochemical potency and specificity, BC11-38 and BC11-38-1 represent logical starting points for medicinal chemistry approaches for developing compounds suitable for whole animal studies of PDE11 function. Such compounds could produce an acute loss of PDE11 activity as a way of identifying biological roles for PDE11, which might be overlooked in
knock-out mouse studies due to compensation of activity by other PDEs or developmental alterations caused by an early loss of PDE11 activity.

The PDE11-specific inhibitors identified in this study could also serve as lead compounds to develop therapeutics to treat adrenal insufficiencies that result in cortisol deficiency. In the adrenocortical cells, binding of ACTH to its receptor leads to cAMP-dependent activation of PKA (Lacroix et al., 2001). Transcription of several genes that express the steroidogenic enzymes involved in cortisol biosynthesis are regulated by transcription factors that are activated by PKA (Gardner et al., 2011; Lacroix et al., 2001; Rosenberg et al., 2002a; Wang et al., 2000). PKA also directly activates various enzymes that regulate the early steps of steroid synthesis, such as the cholesteryl ester hydrolyase (CEH) enzyme that enhances the formation of free cholesterol from cholesteryl esters (Gardner et al., 2011). Adrenal insufficiency can result from either the destruction or dysfunction of the adrenal cortex, such as in Addison’s disease, or secondary to deficient pituitary ACTH secretion, which is most commonly caused by glucocorticoid therapy (Gardner et al., 2011). Introducing excess cortisol to the patient by a foreign route as part of a glucocorticoid therapy causes a negative feedback regulation of ACTH production from the pituitary glands, which leads to further loss of adrenal gland function. This results in insufficient cortisol production when the patient is taken out of glucocorticoid therapy (Gardner et al., 2011). PDE11-specific inhibitors could be used as a substitution or a supplement to cortisol therapy to stimulate cortisol production by the adrenal glands. This approach could prevent further loss of adrenal
function, and therefore could be therapeutically-preferable to the current practice of administering excess cortisol to the patients. The use of PDE11-specific inhibitors could also be beneficial in the treatment of primary adrenocortical insufficiencies. Autoimmune diseases are the leading cause of primary adrenocortical insufficiencies, such as Addison’s disease, and result in a reduction in the number of functional adrenocortical cells that produce cortisol (Gardner et al., 2011). PDE11-specific inhibitors could provide therapeutic benefit by elevating cortisol production by the remaining cells, leading to an overall increase in the amount of cortisol released from the adrenal glands.

The lack of a biological effect in mammalian cells by the other PDE11-inhibiting compounds does not argue that the biological effect of BC11-38 is due to an off-target activity, since it is most likely related to problems with entry of the compounds into mammalian cells, either due to poor solubility or being bound up by components of the growth media. The 5FOA growth media used in the yeast-based assays is more acidic (pH ~5.0) than tissue culture media (pH ~7.0), which might contribute to the differences in the solubility of the compounds in yeast-based versus mammalian assays. Indeed, compound crystals were observed in the cell culture media in wells containing the three ineffective compounds, indicating solubility problems. Therefore, these compounds could be improved for their solubility and pharmacokinetic properties by medicinal chemistry, which will prepare them for uses in future mammalian cell culture and animal studies, as other potent and selective PDE11 inhibitors.
Increasingly, yeast-based HTSs are being used to discover compounds for the study and treatment of human disorders. This study demonstrates the use of a fission yeast-based screen to identify potent and selective human PDE11 inhibitors, therefore also highlights the use of the fission yeast *Schizosaccharomyces pombe* as a host for the screening of large small-molecule libraries to discover potent, selective, and biologically-active PDE inhibitors. The ability to use a positive growth selection in a 384-well format suggests that other HTSs based on the repression of gene expression could be carried out in *S. pombe* using a similar approach. This study serves as a “proof of principle” that the yeast-based platform described in this thesis can be used in chemical screens to identify potent and selective PDE inhibitors that may be effective in cell culture studies even prior to medicinal chemistry efforts. Such specific compounds will be powerful tools to enhance our understanding of PDEs and to develop therapeutics for several diseases related to cyclic nucleotide signaling defects.

In this thesis, the discovery of four potent PDE11-selective inhibitors using a yeast-based HTS platform is presented. These are the first PDE11-selective inhibitors to be reported. I used these compounds to demonstrate a biological role of PDE11 in cortisol production by adrenocortical cells. BC11-38, along with two structural derivatives, increases cAMP and cortisol levels in human adrenocortical cells, consistent with gene association studies that link PDE11 activity to adrenal function. The disease phenotypes linked to inactivating mutations in *PDE11A* gene are observed using pharmacological inhibitors of the enzyme in adrenocortical cells, thus phenocopying a genetic disorder
using small molecules. These compounds can immediately serve as novel chemical tools to study PDE11 function in cell culture, and as leads to develop therapeutics for the treatment of adrenal insufficiencies. My results further validate this yeast-based HTS platform for the discovery of potent, selective and biologically-active PDE inhibitors.
CHAPTER SIX

REFERENCES


glucose and cyclic AMP signaling, cell wall integrity, and septation. Eukaryot Cell 1, 558-567.


APPENDIX A

STUDIES ON ACTIVITY AND INHIBITION OF PDE4 ISOFORMS USING A YEAST-BASED ASSAY

A.1. INTRODUCTION

The “low Km, cAMP-specific” PDE4 family consists of four subtypes (PDE4A-4D) encoded by four distinct genes, each with multiple alternatively spliced variants that differ in their N-terminal regulatory regions and tissue-specific distribution (Houslay, 2001). PDE4s are characterized by their sensitivity to inhibition by Rolipram.

PDE4 inhibition has been shown to provide a wide range of therapeutic benefits, such as reducing inflammation, enhancing cognition, inhibiting HIV infection, and inducing apoptosis in chronic lymphocytic leukemia (CLL) cells (Angel et al., 1995; Bender and Beavo, 2006; Diamant and Spina, 2011; Lerner and Epstein, 2006; O'Donnell and Zhang, 2004; Rose et al., 2005). However, despite the identification of several PDE4-specific drug candidates over the years, only one PDE4 inhibitor has been FDA-approved for clinical use (Roflumilast for the treatment of chronic obstructive pulmonary disease) (Diamant and Spina, 2011; Spina, 2008). The main obstacle that limits the clinical application of PDE4 inhibitors is the severe side effects associated with the inhibition of PDE4D isoform in the brain, which causes nausea and emesis (Lamontagne et al., 2001; O'Donnell and Zhang, 2004; Robichaud et al., 2002; Spina, 2008). Therefore, current
research efforts are aimed toward identification of isoform-selective compounds that show reduced inhibition of PDE4D compared with other PDE4 subtypes, which would provide therapeutic benefit without causing side effects.

Our lab has developed a fission yeast-based platform for PDE inhibitor screens (Demirbas et al., 2011a; Ivey et al., 2008), which has already been used for identification of biologically-active PDE4-, PDE7-, PDE8-, and PDE11-selective inhibitors. The yeast-based platform takes advantage of genetically engineered yeast cells whose growth behavior reflects the activity of heterologously-expressed PDEs. The cells carry a ura4 reporter construct that is counterselectable for growth in medium containing 5-fluoroorotic acid (5FOA), and is regulated by PDE activity. Cells lacking adenylate cyclase and PDE activity respond to low levels of exogenous cAMP or cGMP to repress ura4 expression, conferring 5FOA-resistant (5FOA\(^R\)) growth. Expression of a PDE increases the amount of exogenous cyclic nucleotide required to confer 5FOA\(^R\) growth due to their hydrolysis by the active enzyme. The addition of a PDE inhibitor allows lower cyclic nucleotide concentrations to confer 5FOA\(^R\) growth, enabling the identification of such compounds using a growth assay in 5FOA medium. This assay is also amenable for high throughput screens (HTSs). The difference in the concentration of cyclic nucleotide required to confer 5FOA\(^R\) growth when the PDE is active versus inactive is compared to assess the cAMP- and cGMP-hydrolyzing activity of the enzyme.
The overwhelming majority of the compounds identified in the yeast-based screens have been confirmed for their potency and selectivity using *in vitro* enzyme and mammalian cell culture-based assays. However, a number of compounds that appear to be PDE inhibitors in *in vitro* enzyme assays do not show the same pattern of inhibitory activity in yeast-based assays. Various studies have noted that the kinetic behavior of a recombinant PDE enzyme does not entirely overlap with that of its endogenous native counterpart (Francis et al., 2011). Here, I present the identification of a PDE4A/4B-specific inhibitor that does not inhibit PDE4D in the yeast-based assay, but is not subtype-selective as judged by *in vitro* enzyme assays.

A.2. MATERIALS AND METHODS

A.2.1. Yeast strains and growth media

Yeast strains used in this study are listed in Table A1. Yeast cells were grown and maintained as described in Chapter Two.

A.2.2. 5FOA growth assays

5FOA growth assays were performed as described in Chapter Two.

A.2.3. Construction of a yeast strain that expresses human PDE4A1

Human *PDE4A1* (RefSeq: U68532) open reading frame was integrated into the chromosome under the *cgs2* promoter in two steps. The first step involved integration of the gene into a plasmid by PCR amplification using oligonucleotides that provide
targeting sequences to the *S. pombe* *cgs2* PDE gene, and incorporation into the pKG3-ura4 plasmid, which carries *cgs2* gene disrupted with *ura4*, by gap repair transformation. A restriction fragment from this plasmid with approximately 500 bp of *cgs2* sequences flanking the *PDE4A1* gene was used to insert *PDE4A1* into *cgs2* locus in JZ666 strain by homologous recombination. Integration of the gene was confirmed by PCR. Genetic crosses and tetrad dissection were performed to introduce *fbp1-ura4* and *fbp1-lacZ* reporters, along with mutations in the glucose/cAMP signaling pathway and a deletion of the *pap1* transcriptional activator gene, which is required for ABC transporter expression, to enhance compound sensitivity.

A.2.4. Gap repair transformation

An overnight culture of cells were grown to log phase in YEL, diluted 1:2 in YEL, grown for 3-4 additional hours, and harvested by centrifugation. Cells were washed twice with dH2O, once with 1x LiAC/TE, and were resuspended in LiAC/TE to a density of ~10^8 cells/ml. 2 μl of denatured salmon sperm DNA, 3 μl of StuI-cut (linearized) pKG3-ura4 plasmid, and 10 μl of concentrated PCR product were added to 100 μl of cell suspension, and incubated at room temperature for 10 min. 260 μl 40% PEG/LiAC/TE was added and the cell suspension was incubated at 30°C for 1 h. Cells were then treated with 43 μl DMSO, heat shocked at 42°C for 5 min, pelleted gently, resuspended in 500 μl dH2O, and plated out on EMM-leu selective media.
A.2.5. Plasmid rescue from yeast cells

Plasmid rescue from yeast cells was performed using the Smash and Grab method of Hoffman and Winston (1987) (Hoffman and Winston, 1987).

A.2.6. Bacterial strains and growth media

ElectroTen-Blue electroporation-competent *E. coli* cells were used for plasmid amplification. Bacteria were grown in LB medium at 37°C.

A.2.7. Transformation into *E. coli* and plasmid purification

10 µl of ElectroTen-Blue electroporation-competent cells were mixed with 1-2 µl of DNA in 88 µl dH₂O. Cells were electroporated in 1 mM gap electroporation cuvettes under 2250 Volts, 22.5 kV/cm, 200 ohm, 25 µF, and mixed immediately with 900 µl LB medium. The cells were then incubated at 37°C for 90 min, and plated out on LB-Ampicillin selective media.

For plasmid purification, single colonies of *E. coli* were inoculated into LB medium and cultured overnight at 37°C. Plasmids were isolated from cells using QIAPrep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions.
Table A1: Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>JZ666</td>
<td>$h^{00}$ ade6-M216 leu1 ura4-D18 cgs2::ura4+</td>
</tr>
<tr>
<td>CHP1452</td>
<td>$h^+$ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1Δ::ura4− cgs2::PDE4A1 git2-2::his7+</td>
</tr>
<tr>
<td>CHP1401</td>
<td>$h^-$ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1Δ::ura4− cgs2::PDE4B2 git2-2::his7+</td>
</tr>
<tr>
<td>CHP1453</td>
<td>$h^-$ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1Δ::ura4− cgs2::PDE4D3 git2-2::his7+</td>
</tr>
<tr>
<td>CHP1167</td>
<td>$h^-$ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1Δ::ura4− cgs2::PDE4D3</td>
</tr>
<tr>
<td>CHP1269</td>
<td>$h^-$ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1Δ::ura4− cgs2::PDE4B2</td>
</tr>
</tbody>
</table>
A.2.8. Transformation into yeast for gene integration into chromosome
Transformation of target DNA with flanking sequences homologous to the intended locus of integration was performed as described for gap repair transformation. Following transformation, cells were grown overnight in YEL, and plated onto 5FOA media.

A.2.9. Strain mating and tetrad dissection
Matings were performed by mixing and growing parent strains on MEA media. The cells were then transferred onto a YEA plate and zygotic asci were individually separated from other cells using the tetrad dissector microscope. The cells were incubated at 37°C for 2-3 hours for breaking of the ascus wall. Each spore from a single zygotic ascus was placed apart from each other on the plate in an orderly fashion, and grown at 30°C until visible colonies were formed. The tetrads were scored for the selected phenotypes by replica-plating onto selective media.

A.2.10. In vitro PDE enzyme assays
In vitro enzyme assays were conducted via the Ba(OH)₂ precipitation method of Wang et al. (Wang et al., 2005), using recombinant human PDE4B and PDE4D enzymes (BPS Biosciences), in the presence of 200 nM cAMP. Inhibitor concentrations that reduce enzyme activity by 50% (IC₅₀) are presented. The values are means of three independent experiments.
A.2.11. cAMP assays

CHP1167 and CHP1269 cells that have no defects in components of the cAMP signaling pathway were used for intracellular cAMP measurements. Cells that were grown to exponential phase in EMM complete medium were treated with 20 µM compounds in the same medium for 1.5 h at 30°C, and harvested using glass-fiber filters. cAMP levels were measured as described by Byrne and Hoffman (Byrne and Hoffman, 1993), using cAMP ELISA kit (Enzo). cAMP levels were normalized to protein content measured using a BCA Protein Assay Kit (Pierce).

A.3. IDENTIFICATION OF SUBTYPE-SELECTIVE PDE4A/4B INHIBITORS VIA A YEAST GROWTH-BASED ASSAY SYSTEM

A.3.1. Identification of subtype-specific PDE4A and 4B inhibitors using yeast growth assays

In order to characterize the inhibitor sensitivity of human PDE4A, I first created a yeast strain that expresses the human PDE4A1 enzyme. This strain was constructed by integration of the PDE4A1 gene into JZ666 strain. Following transformation, iodine staining was performed for colonies grown on EMM-leu media to identify the cells that have PDE activity. The presence of PDE activity increases mating of cells (Figure A1), which is detected by increased iodine staining. The cells which have integrated PDE4A1 were confirmed by PCR and sequencing, and were mated with appropriate strains for integration of fbp1-ura4 and fbp1-lacZ reporters, as well as the pap1- and gpa2- alleles.
HTSs against mouse PDE4A1, rat PDE4A5, and mouse PDE4B3 have been previously carried out by members of the Hoffman lab in an attempt to identify PDE4 inhibitors using yeast cells that express each one of these isoforms (Demirbas et al., 2011a; Demirbas et al., 2011b; Ivey et al., 2008). In order to identify subtype-selective inhibitors of PDE4A and PDE4B, I tested candidate hit compounds from these screens in 5FOA growth assays, using strains that express human PDE4A1, PDE4B2, PDE4D2, and PDE4D3. This led to the identification of BC27 that confers 5FOA\(^R\) growth to strains expressing PDE4A and PDE4B, but not PDE4D (Figure A2). To identify more potent and selective inhibitors, I analyzed compounds that are structurally similar to BC27. One of these structural analogs, BC27-5, is more potent and selective than BC27 for PDE4A/4B inhibition, and does not confer 5FOA\(^R\) growth to the PDE4D-expressing strain up to 50 \(\mu\)M (Figure A2).

The structures of BC27, BC27-5, and Rolipram are presented in Figure A3A. To confirm the potency and selectivity of BC27-5, in vitro enzyme assays were performed using recombinantly expressed and purified PDE4B and PDE4D enzymes. Surprisingly, BC27-5 inhibited both isoforms with similar potencies in vitro (Figure A3B). The difference in the effect of BC27-5 in 5FOA growth vs. in vitro enzyme assays raised two possibilities: 1) the strain expressing PDE4D required a greater degree of enzyme inhibition for 5FOA\(^R\) growth, or 2) the in vitro structure and activity of PDE4D has differences from its
behavior *in vivo*, so that BC27-5 inhibits the enzyme in *in vitro* enzyme assays, but not in yeast cells.

In contradiction to the first hypothesis, the PDE4-specific inhibitor Rolipram inhibits all PDE4 subtypes in 5FOA growth assays, and is most effective on the PDE4D-expressing strain (Figure A2). In addition, while characterizing the structural derivatives of a previously identified PDE4-inhibitor, BC69, a compound with PDE4D-selective activity in 5FOA growth assays was discovered. While BC69 inhibits all three PDE4 isoforms, its structural analog BC69-3 inhibits only PDE4D in yeast growth assays (Figure A4). BC69-3 is also selective for PDE4D in *in vitro* enzyme assays (Figure A5B). These results demonstrate that PDE4D inhibition can be detected in 5FOA growth assays, and the lack of an effect by BC27-5 on PDE4D in yeast growth assays is inconsistent with a higher activity of this enzyme compared to PDE4A and PDE4B in *S. pombe*.

5FOA\(^R\) growth is achieved via PKA-mediated transcriptional repression of the *fbp1-ura4* reporter, and thus serves as a reflection of intracellular cAMP levels. To confirm that these compounds act via PDE inhibition in yeast cells, I measured the change in cAMP levels in cells after 1 h compound treatment. Consistent with the 5FOA growth assay results, BC27-5 elevates cAMP levels in PDE4A- and PDE4Bexpressing cells, while BC69-3 elevates cAMP levels in PDE4D-expressing cells only (Figure A6). Rolipram and BC69 increase cAMP levels in all three strains. These results demonstrate that 5FOA
growth assays reflect the PDE inhibition profile of compounds, and the lack of an effect by BC27-5 on PDE4D strain in these assays is not due to an artifact in any downstream event following the increase in cAMP levels.

In order to test whether BC27 would have a synergistic effect with Rolipram for PDE4D inhibition, I performed 5FOA growth assays in the presence of 0.25 µM Rolipram, which provides a low level of PDE4D inhibition that is detectable in these assays (Figure A7). In these conditions, BC27 moderately enhances inhibition of PDE4D cAMP-hydrolyzing activity by Rolipram. This suggests that BC27 is able to bind to PDE4D in yeast cells, however its affinity for the enzyme might be too low to be detected in 5FOA growth assays when the enzyme is fully active. 0.25 µM Rolipram decreases enzyme activity to a lower level, allowing the observation of the low degree of inhibition by BC27.
**Figure A1: Construction of a yeast strain that expresses human PDE4A1 gene.** (A) Human *PDE4A1* gene PCR product was integrated by homologous recombination into the *cgs2* locus in the JZ666 strain, which had been disrupted with *ura4* to allow detection by 5FOA counterselection. (B) Cells lacking PDE activity have constitutively high cAMP levels and cannot sporulate in glucose-starved conditions. Introduction of a functional PDE allows downregulation of intracellular cAMP levels, which enables cells to sporulate under glucose-starved conditions.
Figure A1

A

B

No PDE

PDE4A activity
Figure A2: BC27 and BC27-5 selectively inhibit PDE4A/4B in yeast growth assays.

Profiling of BC27, BC27-5 and Rolipram in 5FOA growth assays were performed against strains that express PDE4A (square), 4B (circle), or 4D (triangle) isoforms. Dose-response growth curves represent the average values from three independent experiments (each with four wells for each data point) ±S.E.M.
Figure A2

[Graphs showing optical density changes with [BC27] and [BC27-5] concentrations.]

[Graph showing optical density changes with [Rolipram] concentrations, with different PDE4 isoforms represented by different symbols.]
Figure A3: BC27 and BC27-5 are not subtype-selective in *in vitro* enzyme assays. (A) Structures and (B) IC$_{50}$ values of the inhibitors in *in vitro* enzyme assays. Values represent averages of three independent experiments, each carried in triplicate. BC27 and BC27-5 inhibit both PDE4B and PDE4D with a similar potency in *in vitro* enzyme assays.
Figure A3

A

BC27

BC27-5

Rolipram

B

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>Rolipram</td>
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<tr>
<td>PDE4B</td>
<td>41.2</td>
</tr>
<tr>
<td>PDE4D</td>
<td>47.3</td>
</tr>
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</table>
**Figure A4: BC69-3 selectively inhibits PDE4D in yeast growth assays.** Profiling of BC69 and BC69-3 in 5FOA growth assays were performed against strains that express PDE4A (*square*), 4B (*circle*), or 4D (*triangle*) isoforms. Dose-response growth curves represent the average values from three independent experiments (each with four wells for each data point) ±S.E.M.
Figure A4

![Graph showing optical density vs. [BC69] (µM) for PDE4A, PDE4B, and PDE4D.](image)

![Graph showing optical density vs. [BC69-3] (µM) for PDE4A, PDE4B, and PDE4D.](image)
Figure A5: BC69-3 selectively inhibits PDE4D in *in vitro* enzyme assays. (A)

Structures and (B) IC$_{50}$ values of BC69 and BC69-3 in *in vitro* enzyme assays. Values represent averages of three independent experiments, each carried in triplicate. BC69-3 is selective for PDE4D in both 5FOA growth and *in vitro* enzyme assays.
Figure A5

A

BC69

BC69-3

B

<table>
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<tr>
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<tr>
<td></td>
<td>BC69</td>
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<tr>
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<td>222</td>
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<tr>
<td>PDE4D</td>
<td>48.2</td>
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</table>
**Figure A6:** The ability of the compounds to elevate cAMP levels in yeast cells is consistent with their ability to confer 5FOA-resistant growth. cAMP levels were measured in cells following a 1.5 h-treatment with 20 µM compound. Results are presented as fold of DMSO control. Values represent averages of three independent experiments (each carried in duplicate) ±S.E.M.
Figure A6

PDE4B

PDE4D

[Graph showing cAMP levels for different conditions]
Figure A7: BC27 moderately increases PDE4D inhibition by Rolipram. 20 µM BC27 alone does not inhibit PDE4D in 5FOA growth assays, however it has a synergistic effect for enzyme inhibition with 0.25 µM Rolipram. Average values from three independent experiments are presented (each with four wells for each data point) ±S.E.M.
Figure A7

PDE4D3

Optical Density

[cAMP] (mM)

- No compound
- 0.25 μM Rolipram
- 0.25 μM Rolipram + 20 μM BC27
- 20 μM Rolipram
- 20 μM BC27
A.4. CONCLUSIONS AND FUTURE DIRECTIONS

5FOA growth assay results suggest that BC27 and BC27-5 are specific for PDE4A and PDE4B, and have little to no activity against PDE4D. However, *in vitro* enzyme assays suggest that BC27 and BC27-5 inhibit PDE4D with the same potency as they display against PDE4B, and with greater potency than Rolipram against PDE4D. Inhibition of PDE4D by Rolipram in yeast-based assays indicates that PDE4D inhibition can be observed using this method. In addition, the identification of BC69-3, a compound that specifically inhibits PDE4D in both 5FOA growth and *in vitro* enzyme assays, demonstrates that the lack of an effect by BC27 and BC27-5 on PDE4D in 5FOA growth assays is not due to a requirement of a greater degree of enzyme inhibition for 5FOA\(_R\) growth by the PDE4D-expressing strain. Measurement of intracellular cAMP levels in the presence of compounds demonstrates that the lack of PDE4D inhibition by BC27-5 in 5FOA growth assays is not due to an artifact in any downstream event following the increase in cAMP levels. This observation further supports the hypothesis that these assays provide a reliable indication for the inhibitory profile of compounds. These results raise the possibility that the structure and activity of PDE4D in *in vitro* enzyme assays might have differences from its *in vivo* behavior. *In vitro* assays are performed in conditions that are significantly different from the intracellular environment, which might affect enzyme structure and activity. These variations, in addition to the differences in substrate and ion concentrations used in *in vitro* versus cell-based assays, might be responsible for the inconsistent results between these methods. Although there are differences between the intracellular environment of yeast cells with
mammalian cells, the majority of inhibitors identified in yeast-based experiments are confirmed in *in vitro* enzyme assays, and several of them show biological activity in mammalian cell culture studies. We have observed more inconsistent results with the PDE4D enzymes, suggesting that these enzymes might have more pronounced differences in their structure and activity in *in vitro* assays. It has been found in several studies that catalytic properties and inhibitor potencies of recombinant PDEs might be different compared to their native counterparts (Francis et al., 2011). Whether the *in vitro* enzyme assays or the yeast-based assays provide a more accurate representation of PDE4D activity in mammalian cells remains to be determined. Experiments to illustrate PDE4D inhibition in mammalian systems would be very helpful to resolve these issues.
A.5. REFERENCES CITED IN APPENDIX A


APPENDIX B

CHARACTERIZING THE SUBSTRATE–SPECIFICITY OF MAMMALIAN PDES USING A YEAST-BASED ASSAY

B.1. INTRODUCTION

Mammals possess 21 genes encoding 11 PDE families that are distinguished by their substrate specificity, conserved regulatory domains, and sensitivity to various pharmacological agents (reviewed by (Bender and Beavo, 2006; Lerner and Epstein, 2006). According to their substrate-specificity, PDEs are grouped as cAMP-specific (PDEs 4, 7, and 8), cGMP-specific (PDEs 5, 6, and 9) and dual-specific (PDEs 1, 2, 3, 10, and 11), which hydrolyze both cyclic nucleotides with nearly equal efficiency.

In this study, I present the application of our yeast-based platform for characterizing PDEs in live cells (Demirbas et al., 2011a; Demirbas et al., 2011b). By expressing mammalian PDEs under conditions that closely resemble their natural intracellular environment, this system provides an easy and reliable identification of PDE activity. In addition to cAMP, cGMP also activates PKA in S. pombe, which allows the analysis of cGMP-hydrolyzing activity using the yeast growth assay, as well. PDEs are expressed in yeast cells that lack adenylate cyclase activity, due to a mutation in git2+ adenylate cyclase gene. The cyclic nucleotide-mediated growth response of these strains is
compared to that of a strain lacking both adenylate cyclase and PDE activity, to assess the relative activity of the PDE against cAMP and cGMP.

B.2. MATERIALS AND METHODS

B.2.1. Yeast strains and growth media

Yeast strains used in this study are listed in Table B1. Yeast cells were grown and maintained as described in Chapter Two. Strains with mammalian PDEs had the following inserts: human PDE1B1 (RefSeq: NM_000924), human PDE4A1 (RefSeq: U68532), human PDE4B2 (RefSeq: L20971), human PDE4D2 (RefSeq: NM_001197221.1), human PDE4D3 (RefSeq: U50159.1), bovine PDE5A1 (RefSeq: NM_001197221.1), human PDE4D3 (RefSeq: U50159.1), bovine PDE5A1 (RefSeq: NM_001197221.1), human PDE7B1 (RefSeq: NM_018945), and human PDE9A5 (RefSeq: NM_001001570.1).

B.2.2. 5FOA growth assays

5FOA growth assays were performed as described in Chapter Two. Profiling of cAMP- and cGMP-hydrolyzing activity were performed in 5FOA medium with cyclic nucleotide concentrations in a range of from 5 mM to 0.101 µM, by making 2/3 dilutions in 23 consecutive columns. Cells were added in 25 µl to wells containing 25 µl of 5FOA medium with 2x cyclic nucleotide concentration, to achieve 50 µl final volume. Plate incubations and OD measurements were performed as described in Chapter Two.
Table B1: Yeast strains used in this study

<table>
<thead>
<tr>
<th>PDE in the strain</th>
<th>Strain ID</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
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<td>No PDE</td>
<td>CHP1207</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 his7-366 pap1::ura4-cgs2-2 git2-2::his7+</td>
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<tr>
<td>PDE1B</td>
<td>CHP1222</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 his7-366 pap1::ura4-cgs2::PDE1B git2-2::his7+</td>
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<td>PDE4A1</td>
<td>CHP1452</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE4A1 git2-2::his7+</td>
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<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE4B2 git2-2::his7+</td>
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<td>PDE4D3</td>
<td>CHP1453</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE4D3 git2-2::his7+</td>
</tr>
<tr>
<td>PDE4A1(D201A)</td>
<td>CHP1476</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE4A1(D201A) git2-2::his7+</td>
</tr>
<tr>
<td>PDE4B2(D241A)</td>
<td>CHP1521</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE4B2(D241A) git2-2::his7+</td>
</tr>
<tr>
<td>PDE5A</td>
<td>CHP1223</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 his7-366 pap1::ura4-cgs2::PDE5A git2-2::his7+</td>
</tr>
<tr>
<td>PDE7B</td>
<td>CHP1221</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 pap1::ura4-cgs2::PDE7B git2-2::his7+</td>
</tr>
<tr>
<td>PDE9A</td>
<td>CHP1218</td>
<td>h+ fbp1::ura4+ ura4::fbp1-lacZ leu1-32 his7-366 pap1::ura4-cgs2::PDE9A5 git2-2::his7+ git3::kan</td>
</tr>
</tbody>
</table>
B.2.3. Construction of yeast strains that express mutant alleles of PDE4A and PDE4B enzymes

Mutant alleles of PDE4A1 and PDE4B2 were created by two-step PCR, which involves the use of two sets of PCR primers to amplify the gene of interest, each set containing one oligonucleotide with the desired mutation, and joining of the two products into a single product. These PCR products were then integrated into the cgs2 locus by homologous recombination, as described for the wildtype PDE4A1 gene in Appendix A.

B.3. PROFILING THE SUBSTRATE-SPECIFICITY OF PDES USING THE YEAST-BASED PLATFORM

Characterization of the activity of PDE1B, PDE5A, PDE7B, and PDE9A were performed by 5FOA growth assays, using strains lacking adenylate cyclase activity. The behavior of the cells expressing each one of these enzymes was compared to the strain that does not have any PDE (Figure B1). The 5FOA growth assays demonstrate that the cAMP-specific PDE7B hydrolyzes only cAMP, whereas the cGMP-specific PDE5A and PDE9A hydrolyze only cGMP. The dual-specific PDE1B hydrolyzes both cyclic nucleotides with similar efficiency in these assays. These results show that the yeast growth assay serves as a reliable method in classification of PDE activity.

PDE4 enzymes, which are classified as cAMP-specific PDEs, were also profiled for their substrate-specificity in yeast growth assays. Surprisingly, PDE4D shows significant cGMP-hydrolyzing activity in yeast growth assays, indicated by the greater requirement
of cGMP needed to confer 5FOA$^R$ growth compared to the condition where PDE4D is inhibited by Rolipram (Figure B2). This was observed in strains expressing both the short isoform PDE4D2 and the long isoform PDE4D3. The activity of PDE4D creates a requirement for $>5$ mM cAMP and 2.5 mM cGMP for 5FOA$^R$ growth to reach optical density of 0.6, compared to the conditions where the enzyme is inhibited by Rolipram. A less efficient hydrolysis of cGMP was also observed in PDE4B- and PDE4A-expressing strains. The PDE4A-expressing strain needs 1.5 mM cAMP and 50 µM cGMP, while PDE4B-expressing strain needs 4 mM cAMP and 80 µM cGMP for 5FOA$^R$ growth to reach optical density of 0.6.

In parallel to these experiments, I created yeast strains expressing PDE4A and PDE4B isoforms with specific mutations that are known to alter their substrate-specificity and sensitivity to Rolipram (Herman et al., 2000). PDE4A(D201A) and PDE4B(D241A) mutants gain cGMP-hydrolyzing activity while having reduced cAMP-hydrolyzing activity in yeast growth assays, consistent with previous data by Herman et al. (Figure B3). These results suggest that the yeast growth assays accurately reflect the cyclic nucleotide-specificity of the exogenously-expressed PDEs, lending further support to the idea that PDE4D is actually a dual-specific PDE rather than a cAMP-specific PDE.
Figure B1: cAMP/cGMP profiles of PDE1B, PDE5A, PDE7B, and PDE9A. cAMP and cGMP requirements of the PDE-expressing strains were compared to those of the strain with no PDE activity in yeast growth assays. The x-axis indicates the concentration of cyclic nucleotide added to the 5FOA medium. Dose-response growth curves represent the average values from three independent experiments (each with four wells for each data point) ±S.E.M.
Figure B1

**PDE1B**

- No PDE (cAMP)
- PDE1B (cAMP)
- No PDE (cGMP)
- PDE1B (cGMP)

**PDE5A**

- No PDE (cAMP)
- PDE5A (cAMP)
- No PDE (cGMP)
- PDE5A (cGMP)

**PDE7B**

- No PDE (cAMP)
- PDE7B (cAMP)
- No PDE (cGMP)
- PDE7B (cGMP)

**PDE9A**

- No PDE (cAMP)
- PDE9A (cAMP)
- No PDE (cGMP)
- PDE9A (cGMP)
Figure B2: PDE4D has significant cGMP-hydrolyzing activity in yeast cells. 5FOA growth response to exogenous cAMP and cGMP in strains expressing PDE4A, PDE4B, PDE4D2 (short isoform), and PDE4D3 (long isoform) were compared to the conditions where the enzymes were inhibited by 20 µM Rolipram. The x-axis indicates the concentration of cyclic nucleotide added to the 5FOA medium. Dose-response growth curves represent the average values from three independent experiments (each with four wells for each data point) ± S.E.M. >4 mM cGMP is needed for PDE4D-expressing cells to reach an optical density >1.0, while inhibition of the enzyme by Rolipram confers 5FOA<sup>R</sup> growth at <0.2 mM cGMP, indicating hydrolysis of exogenously added cGMP by PDE4D.
Figure B2

PDE4A

PDE4B

PDE4D3

PDE4D2

- cAMP
- cAMP+Rolipram
- cGMP
- cGMP+Rolipram
Figure B3: Yeast growth assays verify cGMP-hydrolyzing activity by PDE4A and PDE4B mutants. (A) Location of the aspartic acid in human PDE4A1 (Genbank accession number U68532) and PDE4B2 (Genbank accession number L20971), whose mutation to alanine confers cGMP-hydrolyzing activity. The aspartic acid corresponds to residue number 201 in PDE4A1 and 241 in PDE4B2. (B) 5FOA growth response to exogenous cAMP and cGMP in strains expressing wild-type and mutant PDE4A and PDE4B. The x-axis indicates the concentration of cyclic nucleotide added to the 5FOA medium. Dose-response growth curves represent the average values from three independent experiments (each with four wells for each data point) ±S.E.M.
Figure B3

A

PDE4A1: LHAA\textbf{D}VLQS
PDE4B2: LHAA\textbf{D}VAQS

B

![Graph showing optical density against cyclic nucleotide concentration for PDE4A and PDE4B isoforms with different nucleotides.

- PDE4A-cAMP
- PDE4A-cGMP
- PDE4A(D201A)-cAMP
- PDE4A(D201A)-cGMP

- PDE4B-cAMP
- PDE4B-cGMP
- PDE4B(D241A)-cAMP
- PDE4B(D241A)-cGMP]
B.4. CONCLUSIONS AND FUTURE DIRECTIONS

The yeast-based assay accurately characterizes the substrate-specific activity of the cAMP-specific PDE7, cGMP-specific PDE5 and PDE9, and dual-specific PDE1. PDE4A and PDE4B appear highly specific for cAMP-hydrolysis in 5FOA growth assays; however, PDE4D enzymes (both the short and long isoforms) display significant activity for cGMP hydrolysis that is comparable to their cAMP-hydrolyzing activity in yeast cells.

PDE4 enzyme family is classified as cAMP-specific based on data from several in vitro studies. This study suggests that PDE4D enzymes might in fact have significant cGMP-hydrolyzing activity in vivo. Consistent with my observations in yeast cells, Bellamy et al. demonstrated that in cultured cerebellar cells PDE4 enzymes significantly contribute to cGMP degradation at high intracellular cGMP concentrations, and this activity is inhibited by Rolipram (Bellamy and Garthwaite, 2001). These results suggest that Rolipram administration might have significant effects on intracellular cGMP concentrations as well, and this should be taken into consideration in studies of cAMP-related functions.

The results presented in Appendix A suggest that the in vivo activity of PDE4D might be different from its activity in in vitro enzyme assays. The yeast-based assay provides a profiling of substrate-specificity that is consistent with in vitro assays for the majority of PDEs. Figure B3 demonstrates that this assay is sensitive to detect the effects of point mutations that alter the substrate-specificity of enzymes. The intracellular environment
of yeast cells closely resembles that of mammalian cells. Various previous studies have noted differences in the behavior of recombinant PDEs with that of their endogenous counterparts, including their substrate affinity, catalytic efficiency, and inhibitor potencies (Francis et al., 2011). The heterogeneity in the conformation of some recombinant proteins is a major variable contributing to these differences (Francis et al., 2011). It remains to be determined whether in vitro enzyme assays or the yeast-based assays provide a more accurate representation of PDE4D activity in mammalian cells. Future studies that provide a live measurement of PDE activity in mammalian cells would be beneficial for elucidating PDE4D activity in vivo.
B.5. REFERENCES CITED IN APPENDIX B


