

## Article

# Development of Phosphodiesterase-Protein Kinase complexes as novel targets for discovery of inhibitors with enhanced specificity

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**Abstract:** Phosphodiesterases (PDEs) hydrolyze cyclic nucleotides to modulate multiple signaling events in cells. PDEs are recognized to actively associate with cyclic nucleotide receptors (Protein Kinases, PK) in larger macromolecular assemblies referred to as signalosomes. Complexation of PDEs with PK generates an expanded active site which enhances PDE activity. This facilitates signalosome-associated PDEs to preferentially catalyze active hydrolysis of cyclic nucleotides bound to PK, and aid in signal termination. PDEs are important drug targets and current strategies for inhibitor discovery are based entirely on targeting conserved PDE catalytic domains. This often results in inhibitors with cross-reactivity amongst closely related PDEs and attendant unwanted side effects. Here, our approach targets PDE-PK complexes as they would occur in signalosomes, thereby offering greater specificity. Our developed fluorescence polarization assay has been adapted to identify inhibitors that block cyclic nucleotide pockets in PDE-PK complexes in one mode, and disrupt protein-protein interactions between PDEs and cyclic nucleotide activating protein kinases in a second mode. We tested this approach with three different systems: cAMP-specific PDE8-PKAR, cGMP-specific PDE5-PKG and dual-specificity RegA-R<sub>D</sub> complexes and ranked inhibitors according to their inhibition potency. Targeting PDE-PK complexes offers biochemical tools for describing the exquisite specificity of cyclic nucleotide signaling networks in cells.

**Keywords:** Phosphodiesterase (PDE), natural products, inhibitors, Protein Kinase, selectivity, fluorescence polarization

**Abbreviations:** 2fc - 2'-fluoro-AHC-cAMP; 2fg - 2'-fluoro-AHC-cGMP; cAMP - cyclic 3',5' adenosine monophosphate; cGMP - cyclic 3',5' guanosine monophosphate; cNMP - cyclic nucleotide monophosphate; PKAR – Regulatory subunit of Protein Kinase A; R<sub>D</sub> - Regulatory subunit of Protein Kinase A of *Dictyostelium discoideum*; 2fc-PKAR - PKAR bound to 2fc; 2fc-R<sub>D</sub> - R<sub>D</sub> bound to 2fc; 2fg-PKG - Protein Kinase G bound to 2fg; PDE – Phosphodiesterases; PDE-PK - general Phosphodiesterase-Protein Kinase complex

## 1. Introduction

Second messenger cyclic nucleotides (cNMP) are important regulators of numerous cellular pathways. Phosphodiesterases (PDEs) catalyze hydrolysis of cyclic nucleotides (cyclic 3', 5' adenosine monophosphate (cAMP) and cyclic 3', 5' guanosine monophosphate (cGMP)) and regulate overall levels of cyclic nucleotides and thereby impact the magnitude and duration of the cellular response. This makes them important targets for drug discovery [1-3]. In mammals, the PDE superfamily of phosphodiesterases comprises 11 different families, each with numerous subtypes and isoforms [4]. Isoforms of various PDEs have been effectively targeted for treatment of cardiac arrhythmia, inflammation,

erectile dysfunction and steroidogenesis [1]. Based on their substrate specificity PDEs are categorized broadly into cAMP-specific, cGMP-specific and dual specificity PDEs. About 100 PDEs are thus distributed in various tissues and across different stages of development. Each PDE isoform includes a conserved C-terminal catalytic domain associated with one or more variable N-terminal regulatory domains. Association of PDEs with specific receptors and cyclases to form signaling islands referred to as 'signalosomes' has been increasingly recognized to be the primary mode of cyclic nucleotide regulation in cells [5-7]. These signaling islands are mediated by specific scaffold proteins that localize multiple elements of the cNMP signaling pathway [8-14].

Several PDE signalosomes have been reported to regulate various cellular functions [7, 15-17]. In all of these signalosomes, PDEs are anchored in close proximity to cNMP receptors and function as multivalent macromolecular assemblies, rather than as free diffusive PDEs [6, 17]. Consequently, localized PDEs facilitate hydrolysis of cytosolic cNMPs within these assemblies. An imperative effect of such colocalization of PDEs and cNMP receptors is that the PDEs are poised to hydrolyze cNMP bound to the receptors Protein Kinases (PK) [18]. This 'direct' hydrolysis bound cNMPs offers precision in regulating various signaling pathways and imparts specificity by formation of complex. Inhibition of PDEs leads to overall increase in cNMP levels, which is essential in disease control. Targeting the receptor-bound PDE, rather than PDEs alone, for drug discovery forms the rationale of this study and overcomes some of the limitations of nonspecific overlapping effects that inhibitors targeting conserved PDE sites might present. Due to the ubiquitous presence of PDEs in the body, the low selectivity of particular inhibitors to their respective phosphodiesterases gets translated into unintended adverse effects in cells. For example, cyanopsia (i.e. blue tinge in vision) is associated with the use of sildenafil, a PDE5-specific inhibitor. This visual symptom occurs due to its cross-reactivity of sildenafil with PDE6, which is present only in rod and cone photoreceptors [19, 20]. Hence, this necessitates the need for more isoform selective and specific inhibitors to overcome the cross-reactivity problem faced by current PDE inhibitors.

Our goal is to determine the degree of PDE inhibition by different small molecules using PDE-PK complexes as the targets. To screen inhibition of cAMP- or cGMP- specific PDEs, we selectively chose (i) cAMP-specific - PDE8-Protein Kinase A complex (mammalian), (ii) cGMP-specific - PDE5-Protein Kinase G (PKG) complex (mammalian), and (iii) a dual cAMP/cGMP selective RegA-PKA system (from *Dictyostelium discoideum*). cAMP-dependent proteins kinase A (PKA) and cGMP-dependent Protein Kinase G (PKG) represent two of the most important cyclic nucleotide effectors [17, 21]. In PKG, the catalytic kinase domains are expressed with the regulatory domains as a single polypeptide. On the contrary, in PKA, the regulatory and kinase domains are expressed as individual - regulatory (R) and catalytic (C) subunits. There are two non-redundant cyclic nucleotide binding domains (CNB) present in both PKA (R-subunit) and PKG, each with slightly altered cNMP binding affinity. PDE8-PKAR complex is formed by PDE8 dimer binding to each cAMP-R-subunit domains, thus comprising two distinct 'composite active sites' formed by the coupling of the PDE active site with each cyclic nucleotide binding site [6]. The CNB domains on the kinase receptors have high affinities for cAMP (PKAR,  $K_D \sim 2$  nM) and cGMP (PKG,  $K_D \sim 100$  nM) but also bind cyclic nucleotide analogs with high affinities [22-24].

Given that a majority of intracellular PDEs are localized within signalosomes [8, 11, 13], the PDE-PK complexes with two distinct functional composite active sites, and represent relevant high specificity targets for inhibitor discovery. In this study, we propose an assay which leverages the reverse modularity inherent in PDE catalysis wherein the catalytic properties of the PDE active site are directly altered by the cNMP receptor (Protein Kinases) it is bound to. High specificity of inhibitor binding is thus achieved by relying upon the specific functional output responses generated from the distinct PDE-PK active sites [6, 21]. We propose a fluorescence polarization assay for measuring cNMP-specific displacement of fluorescent cAMP/cGMP analogs from PDE-PK complexes. This allows

us to screen natural product extracts for potential PDE inhibitory activities and to subsequently identify specific inhibitors. Using known PDE inhibitors, we assessed the sensitivity and applicability of our approach, and compared with a commercially available phosphodiesterase assay. Our results reveal that composite active sites of PDE-PK complexes offer a great tool in identification of inhibitors with improved specificity for cAMP/cGMP active sites and selectivity for single or dual sites. This developed method enables screening of novel PDE inhibitors that specifically target different classes of cNMP targets and thereby offers a new route for accelerating discovery of target receptor-specific PDE inhibitors.

## 2. Results

### 2.1 *Designing competitive cNMP-dependent displacement assay for PDE inhibitors*

We first set out to assess a Phosphodiesterase-Protein Kinase complex as an enzymatic tool to monitor the hydrolysis of cNMP substrates and its displacement by small molecules. To monitor real-time association or dissociation of cyclic nucleotides, we used fluorescent analogs of cAMP (2fluo-cAMP, 2fc) and cGMP (2fluo-cGMP, 2fg), which reversibly bind to PKAR, R<sub>0</sub> and PKG with high affinities. We first measured the kinetic complexation of catalytic domains of PDEs with their specific Protein Kinases and tested their stability and catalytic activities using cAMP or cGMP substrates.

#### 2.1.1 Designing a cAMP-specific assay using PDE8-PKAR complex

For testing small molecules inhibiting cAMP-specific PDEs, we used mammalian PDE8-PKAR system. FP of 2fluo-cAMP-bound PKAR ('2fc-PKAR') was constant throughout (Fig. 1 i, blue), indicating their stable binding. Next, PDE8c in the absence or presence of excess cAMP was added at time  $t = 20$  min. Addition of PDE8c lead to immediate increase in FP values (red plot), suggesting formation of complex between PDE8c and 2fc-PKAR, which remained stable over time. Addition of cAMP/PDE8c lead to gradual decrease in FP (orange plot), which indicates competitive displacement of 2fluo-cAMP from PKAR by cAMP. The decrease in FP was followed by rapid increase in FP values corresponding to those measured for 2fc-PKAR-PDE8c rather than 2fc-PKAR. Such a FP trend suggests that the PDE-PKAR composite active site preferentially hydrolyzed unlabeled cAMP first, followed by reassociation of 2fc-PKAR-PDE8c complex.

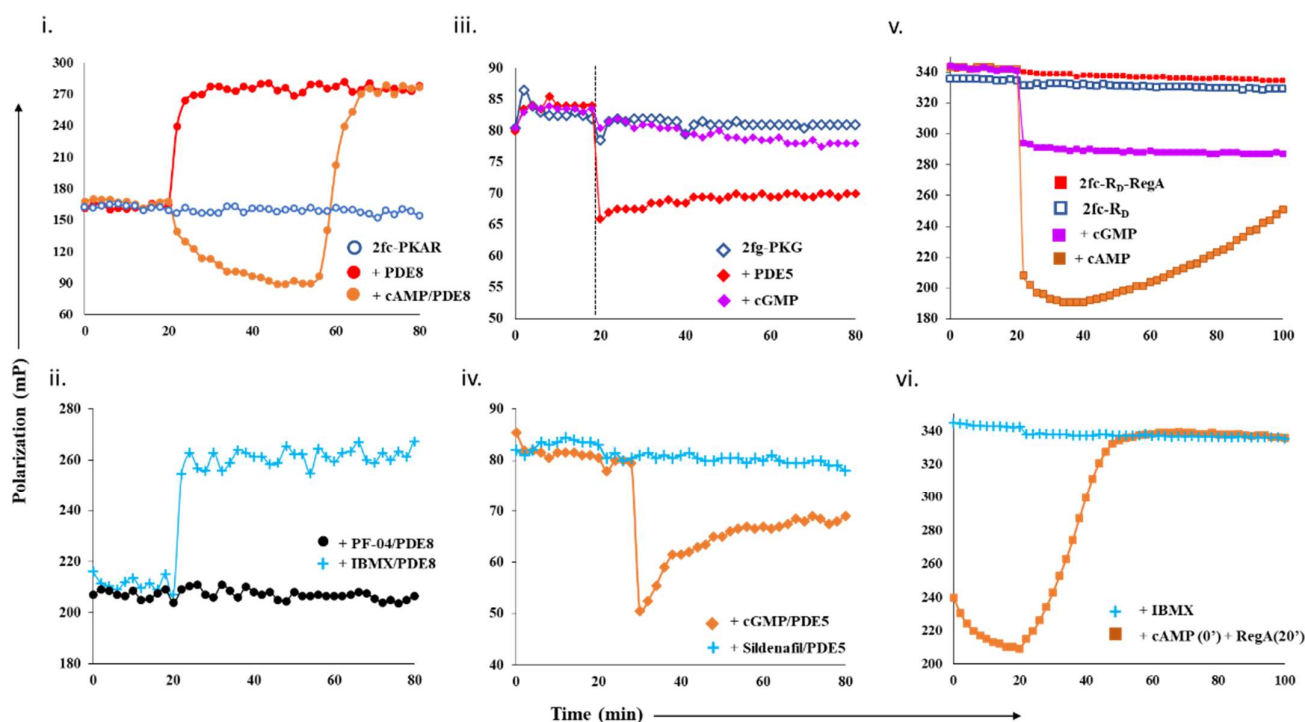


Figure 1: Design and test of competitive displacement assay using PDE-PK complex.

(i, ii) cAMP-specific probe PDE8-PKAR - (i) Active PDE8c (red circles) and cAMP-incubated PDE8c (orange circles) were added to 2fc-PKAR at 20 min and the FP values were measured. (ii) Inhibition and complexation of cAMP-specific PDE8-PK was tested by addition of PDE8c (1  $\mu$ M) incubated with 10  $\mu$ M PF-04957325 (black circles) and 500  $\mu$ M IBMX (cyan plus) to 2fc-PKAR at t = 20 min. (iii, iv) cGMP-specific probe PDE5-PKG - (iii) At 20 min time interval, PDE5 (red diamonds) and cGMP (lilac diamonds) were added to 2fg-PKG (blue circles) and signal was measured for total time course of 80 min. (iv) PDE5 was incubated with substrate 100  $\mu$ M cGMP (orange diamonds) and 1  $\mu$ M Sildenafil (cyan plus) and added to 2fg-PKG at time t = 30 min, to test stability and inhibition of cGMP-specific PDE5-PKG probe. (v, vi) Dual-specificity probe RegA-RD - (v) Plot showing stability of pre-formed composite active sites of 2fc-RD-RegA (red squares) as compared to free 2fc-RD (blue squares). Excess cAMP (orange squares) and cGMP (lilac squares) were added to 2fc-RD-RegA complex at 20 min interval and FP measured for further 80 min. (vi) At t = 20 min, IBMX (cyan plus) was added to 2fc-RD. Separately, cAMP was added to 2fc-RD at time = 0 min, followed by RegA at time = 20 min to this reaction mixture (orange squares) and FP was measured for time course of 100 min.

To use PDE8-PKAR complex as a tool to screen novel inhibitors, we first tested using broad-specific PDE inhibitor IBMX, which binds PDE8 but does not inhibit it and PF-04957325, which is a PDE8-specific inhibitor ( $IC_{50} \sim 1-30$  nM) [25, 26]. PDE8c incubated with IBMX and PF-04957325 was added to 2fc-PKAR to test for activity and complexation (Fig. 1 ii). Consistent with our expectations, we observed that addition of IBMX-saturated PDE8c (cyan plus plots) resulted in increase in FP values similar to those measured for active PDE8c (red plot, Fig. 1 i), indicating that IBMX did not inhibit PDE8c and therefore enabled complexation with 2fc-PKAR. Our FP results showed no reduction in FP for IBMX treated PDE8, suggesting that intracellular PDE8 preferred to be associated with cAMP receptors as composite catalytic site, and therefore preventing PDE8 inhibition by IBMX. This is consistent with literature where IBMX is shown to inhibit other PDEs, but not PDE8, although high-resolution crystal structure showed IBMX bound PDE8 [27, 28]. Addition of PDE8c incubated with PF-04957325 (black circles plot) resulted in no significant change where FP values remained equivalent to 2fc-PKAR (blue open circles, Fig. 1 i). Lack of any increase in FP indicated that inhibited PDE8c was unable to bind to 2fc-PKAR. These results indicate that PDE8-PKAR composite site based fluorescence polarization assay offered a reliable platform to identify inhibitors and distinguish them from small molecules which occupy the catalytic sites.

### 2.1.2 Designing assay for cGMP-specificity using PDE5-PKG complex

Complexation between PDE5 and PKG for cGMP hydrolysis has not been reported yet. Hence, we first monitored the effects of cGMP and PDE5 on 2fg-PKG (Fig. 1 iii, blue diamonds). PDE5 addition lead to instant drop in FP (red plot), while resulted in marginal change in FP values. These results indicate that PDE5 bound PKG transiently to mediate hydrolysis of substrate cGMP, followed by its dissociation and hence resulting in decreased FP. Meanwhile, addition of 1000-fold excess cGMP (lilac plot) resulted in no significant change in FP, indicative that 2fluo-cGMP remained stably bound to PKG.

Subsequently, we tested the displacement of 2fluo-cGMP from PKG by PDE5 in the presence of excess substrate cGMP and inhibitor Sildenafil [19]. Addition of cGMP-saturated PDE5 (orange diamonds, Fig. 1 iv) lead to decrease in FP values greater than that observed for active PDE5 (red diamonds, Fig. 1 iii). This drop in polarization to lower values indicated cGMP-PDE5 displaced 2fluo-cGMP from PKG, suggestive of processive hydrolysis of cGMP by PDE5-PKG composite site. With time, the FP values gradually increased equivalent to that of 2fg-PKG-PDE5 mixture. 2fluo-cGMP gets hydrolyzed by PDE5 and reassociates PKG, thereby leading to increased FP at longer times. On the other hand, addition of Sildenafil-treated PDE5 did not result in any significant changes in FP values, indicative of inhibition of PDE5 and no catalysis. These results therefore highlight the specificity of using PDE5-PKG complex as a tool to differentiate between a kinetically active composite site from an inhibited complex.

### 2.1.3 Designing a dual cAMP/cGMP assay using a broader specificity RegA-R<sub>D</sub> complex

As phosphodiesterase catalytic domains are conserved and show high structural similarity, we used a dual-specific cAMP/cGMP selective PDE which is only partially similar in catalytic domain. Type 2 phosphodiesterase from *D. discoideum*, RegA has high similarity to PDE8 catalytic site and therefore a good choice [29, 30]. Besides, RegA is not specific to cAMP and shows cAMP/cGMP selectivity of ~200, indicating it can bind to both cAMP and cGMP. It has been shown that RegA interacts with its cognate PKA regulatory subunit (R<sub>D</sub>), which also has two cyclic nucleotide binding sites – CNB:A (high affinity) and CNB:B (low affinity) [21]. Here, our strategy was to differentiate small molecules that displace cAMP and/or cGMP.

We monitored the stability of RegA-R<sub>D</sub> complex in the presence of cAMP and cGMP using FP (Fig. 1v). Firstly, in the absence of substrates, FP of 2fc-R<sub>D</sub> (blue square plot) and preformed 2fc-R<sub>D</sub>-RegA complex (red square) remained stable over period of 100 min. We tested the activity of RegA-R<sub>D</sub> composite site by adding cAMP and cGMP at 20 min interval. Addition of cAMP (orange squares) resulted in immediate decrease in FP, indicative of competitive displacement of 2fluo-cAMP by cAMP. However, we observed a gradual and steady increase in FP over the time-course of the assay, suggesting slow reassociation of 2fluo-cAMP to R<sub>D</sub> or RegA-R<sub>D</sub> complex. While cAMP addition lead to complete displacement, we noticed that addition of cGMP (lilac plot) resulted in only partial decrease in FP that remained stable over time. We believe that cGMP displaced 2fluo-cAMP only from one of the two composite sites, probably the low-affinity sites of R<sub>D</sub> and RegA. These results serve as controls for displacement from one (like cGMP) or both (like cAMP) composite sites of RegA-R<sub>D</sub> pair. Next, we monitored the effects of broad-specificity PDE inhibitor IBMX on 2fc-R<sub>D</sub>-RegA complex and observed no change in FP (blue plus plot, Fig. 1 vi). Like cAMP and cGMP, we added cAMP to 2fc-R<sub>D</sub> (orange square plot, Fig. 1 vi) and observed a large decrease in FP, with values similar to those seen above (orange square plot, Fig. 1 v). Addition of RegA to this mixture led to gradual increase in FP. This decrease followed by increased FP indicate dissociation and reassociation of 2fluo-cAMP from RegA-R<sub>D</sub> complex. Hence, we generated enzyme-receptor complex with specificities for



cAMP and/or cGMP and developed a competitive displacement assay using PDE-PK complex as a screening tool to distinguish between cAMP and cGMP specific inhibitors.

## 2.2 *Screening novel inhibitors in plant extracts using competitive displacement assay*

One of the major sources of drugs, especially for phosphodiesterase inhibitors, are natural sources such as plants. A number of natural compounds have been identified as phosphodiesterase inhibitors isolated from medicinal plants and has been reviewed by multiple publications [31-34]. Natural products are intrinsically useful in drug discovery due to their high level of structural as well as chemical diversity.[34] They also possess a unique advantage of having high biochemical specificity and binding affinities to their receptors [35]. In this study, the PDE inhibitory potentials of natural products extracted from two medicinal plant species were investigated using the proposed assay. Both these plants have been shown to possess phytochemicals which inhibit PDEs and other enzymes. We therefore set out to apply PDE-PK complex based fluorescence polarization assay to identify compounds that may bind to interact and have inhibitory potential. As the fluorescent ligands are the reporters, any competition to their binding to PDE-PK complex can be monitored easily and rapidly. Further, known drug molecules that inhibit PDE were selected to test the robustness of this assay and as positive controls.

### 2.2.1 Screening cAMP-specific inhibitors using PDE8-PKAR complex

We next set out to screen plant extracts for PDE inhibition and their effects on PDE-PK complex stability. As phosphodiesterases are clinically important and a major target for drug design, our approach was to test the efficacy of the assay in the presence of known drugs, wherein active PDE8 and PF-04957325-inhibited PDE8 served as negative and positive controls for inhibition. First, PDE8 was incubated with 5  $\mu$ l each of extracts A-F for 15 minutes and then added to wells containing 2fc-PKAR at reaction time 20 min, followed by measuring their FP values. The polarization values observed for extract A-treated PDE8 (Fig. 2 i) were similar to those observed for 2fc-PKAR-PDE8 complex, indicating that the compounds present in this extract were unable to bind or inhibit PDE8 or the PDE8-PKAR composite site. For PDE8 treated with extracts B, D and E, the FP results observed were in-between active or inhibited composite site, indicating that these extracts may possess phytoconstituents that block active site partially (extracts D and E) or gradually (extract B), resulting in a mix of bound and blocked PDE8-PKAR composite sites. Importantly, addition of extract E-treated PDE8 (black cross plot, Fig. 3A) resulted in polarization values similar to PF-04957325 (black circles plot, Fig. 2 i). This suggests that extract E has compounds that bind and blocked PDE8-PKAR composite site formation and thereby inhibiting its activity. These results also show the sensitivity of the assay to screen potent versus weak inhibitors in a single-step procedure. The PDE8-PKAR bound to 2fluorocAMP can therefore be used as a probe to screen small molecules that may either activate or inhibit phosphodiesterases.

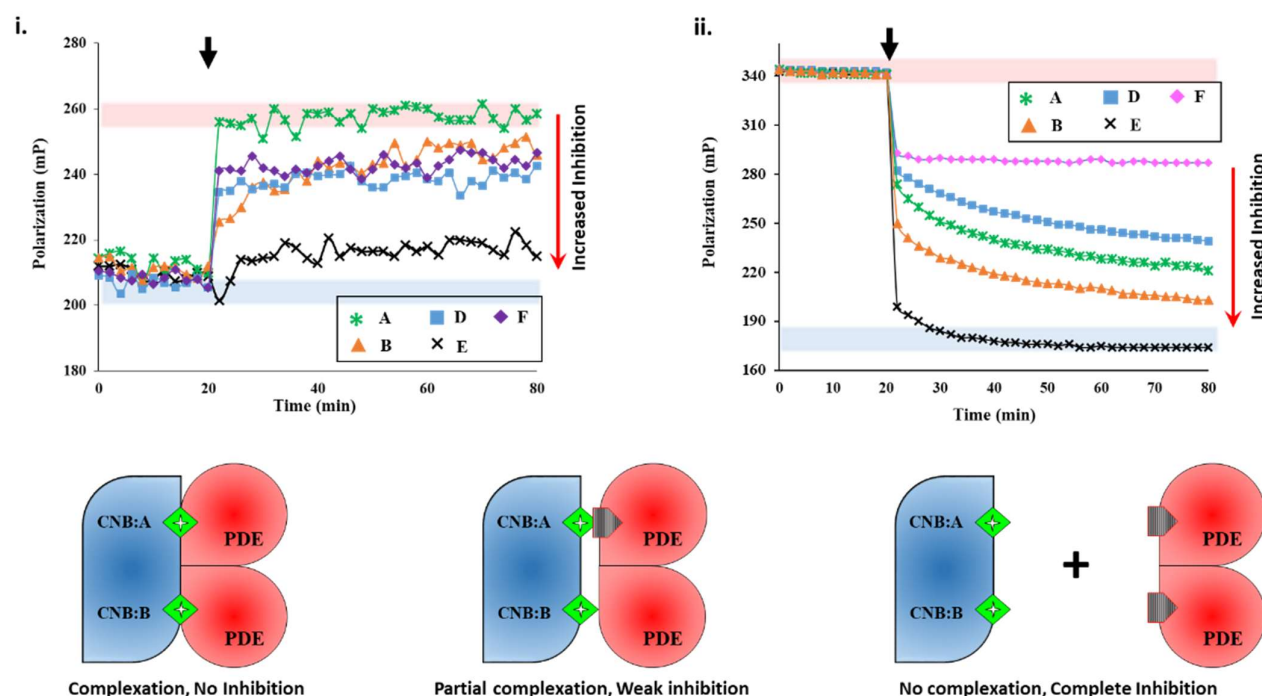


Figure 2: Plant extracts inhibit PDE8 and RegA with different potencies.

(i) Changes in fluorescence polarization (y-axis) over time (x-axis) is represented for 2fluo-cAMP saturated PKAR upon addition of PDE8c pre-incubated with extracts A (green asterisk), B (orange triangles), D (blue squares), E (black cross), and F (lilac diamonds) at 20 min (black arrow). (ii) FP plot showing effect of inhibitors on RegA-Rd complexation. After 20 min (black arrow), 5  $\mu$ l of extracts A, B, D, E, and F were added to preformed 2fc-Rd-RegA complex. Red area indicates zone of no PDE inhibition as per negative control, while blue area indicates high PDE inhibition zone using PDE inhibitors as positive control. (C) 2fluo-cAMP (green diamond) is bound to cyclic nucleotide binding (CNB) domains CNB:A and CNB:B of PKA (blue). Cartoons showing inhibitor (grey pentagon) mediated complete (left), partial (centre) or no (right) complex formation between PDE (red) and PK (blue) due to no, partial or complete PDE inhibition respectively.

### 2.2.2 Screening plant extracts for dual cAMP/cGMP PDE inhibition of the RegA-Rd complex

We next screened the inhibition potency of the plant extracts against cAMP and/or cGMP binding phosphodiesterases. Here, we added the crude plant extracts to RegA-Rd complex and monitored real-time displacement of 2fluo-cAMP. After a baseline read for 20 min, various plant extracts were added to the preformed 2fc-Rd-RegA complex and their FP was recorded for additional 80 min. Amongst the various plant extracts tested, certain crude extracts showed no change in FP values for RegA-Rd pair (data not shown), while extracts A-F showed significant decreases in polarization values (Fig. 2 ii).

Closer inspection of the plots highlighted differences in the relative decrease in polarization between the various plant extracts, indicating that these compounds showed dissimilar inhibitory effects. Addition of extract E (black cross, Fig. 2 ii) to 2fc-Rd-RegA lead to rapid displacement of 2fluo-cAMP with dissociation kinetics faster than that observed for the natural ligand cAMP. This result suggests that extract E is highly potent and has phytoconstituents that bind to the PDE-PK composite site with higher affinity than cAMP. Addition of extract A showed gradual decrease in FP values, although not as potent as extract E, indicating extract A inhibited RegA-Rd. Interestingly, extract A showed no inhibition of PDE8 which suggests its variability in inhibition of cAMP-PDEs. This result is indicative of the robustness of our approach to screen compounds that inhibit PDEs selectively. Furthermore, addition of extract F (Fig. 2 ii) led to displacement only from one site, as the FP values were similar to those observed for cGMP (Fig. 1 v).

The potency or ability of these compounds as potential inhibitors in decreasing order was observed to be extract E>B>A>D>F, where extract E showed complete and rapid displacement of 2fluorocAMP, while extract F had least effect. These results highlight the importance of targeting PDE-PKAR complex as the 'new active site', as it offers insights about the specificity and selectivity of particular compound. Moreover, this also shows that different extracts bind to PDE-PKAR complex with different affinities and have variable inhibitory potencies.

### 2.2.3 Screening cGMP-specific inhibitors by targeting PKG-PDE5 complexes

Next, we tested 2fluorocGMP saturated cGMP-dependent protein kinase G (PKG) with stoichiometric ratios of PDE5 as the composite active site. Previous studies have shown that PKG phosphorylates the N-terminal GAF domain of PDE5 and activates it [36]. However, here the two cGMP-binding sites of PKG, CNB:A and CNB:B, serve as receptors to bind cGMP and interact with PDE5. Addition of PDE5 to 2fg-PKG lead to immediate decrease in polarization values (Fig. 1 iii), indicating dissociation of 2fluorocGMP from PKG, followed by separation of the two proteins. Unlike complex formation between PKAR and PDE8, PDE5 interacts with PKG only to access the substrate followed by dissociation of the ligand, as observed previously [6]. PDE5 treated with Sildenafil was used as a positive control to measure PDE5 inhibition.

PDE5 was treated with various crude plant extracts A-F for 15 min and then added to 2fc-PKG (Fig. 3 i). Amongst various crude extracts tested, only extract B was unable to inhibit PDE5A, while other extracts strongly inhibited PDE5. Addition of PDE5 treated with extract B (orange triangles) resulted in decreased FP values, similar to active PDE5, while PDE5 treated with other extracts showed no significant decrease in the polarization values and were comparable to results obtained for Sildenafil. These observations suggest that extract B was unable to inhibit PDE5, but other extracts likely possessed compounds that inhibit PDE5 and the PDE5-PKG composite site. The inhibition potency of these extracts was F=C>A>D>E in decreasing order. These values were similar to those observed by PDE-Glo™ phosphodiesterase assay.



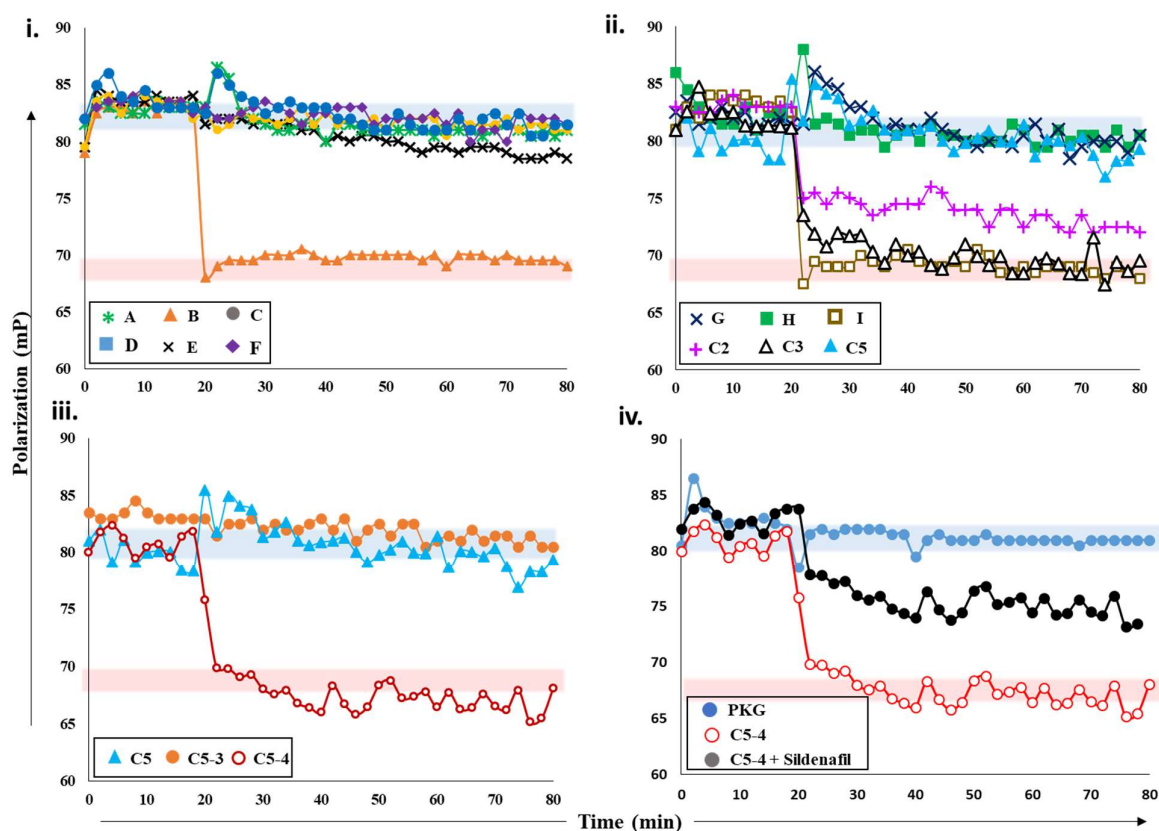


Figure 3: Effect of plant extracts and fractions on PDE5 activity.

(i) Fluorescence polarization versus time plot depicting the changes in FP values of 2fg-PKG upon addition of PDE5 (0.2 μM) incubated with crude plant extracts A-F at 20 min time interval (black arrow). Red zone indicates values similar to active PDE5 (no inhibition), while blue zone indicates FP values similar to inhibited PDE5. Increased FP indicates greater PDE inhibition (red arrow). Inhibition of PDE5 incubated with fractions C2, C3, C5 from crude extract C; sub-fractions C5-3 and C5-4 (panel iii); and pure compounds G, H and I (panel ii). Red area indicates zone of no PDE inhibition based on negative control active PDE5, while blue area indicates high PDE inhibition zone based on positive control Sildenafil. (iv) PDE5 was incubated with sub-fraction C5-4 was spiked with 100 nM Sildenafil (black circles) and added to 2fg-PKG (blue circles) at time  $t = 20$  min. Sub-fraction C5-4 which does not inhibit PDE5 is shown for reference.

Crude extract C was then subjected to fractionation and separation into less complex mixtures. This yielded five fractions C1, C2, C3, C4 and C5 that were then tested for their inhibition of phosphodiesterases (Supplementary Fig. S1). PDE5A incubated with extracts C2, C3 and C5 was added to 2fg-PKG and their polarization values recorded (Fig. 3 ii). Addition of C2-PDE5A lead to decrease in FP values intermediate to those of active and inhibited PDE5A, indicating only partial/weak inhibition. C3-PDE5A addition lead to greater decrease in FP with values similar to active PDE5A indicative of no PDE inhibition. Fraction C5 (blue triangle) showed the greatest PDE5A inhibition as observed by no change in FP when mixed into 2fg-PKG.

Subsequently, fraction C5 was subjected to further fractionation to isolate the active compound(s). Fractions C5-3 and C5-4 obtained were then targeted. PDE5A treated with extract C5-4 addition resulted in decrease in FP (maroon, open circles) with values similar to those of active PDE5A. Fraction C5-3 treated PDE5A addition lead to significant change in FP, with values similar to those observed for 2fg-PKG. These results indicate that sub-fraction C5-4 did not inhibit PDE5A, while C5-3 inhibited PDE5A alike Sildenafil. Furthermore, preliminary mass spectrometry analysis of C5-3 fraction led to identification of few

components which could possibly be PDE5A inhibitors. We then used commercially available standards G, H, and I of the identified small molecules. We applied our competitive displacement assay using PDE5A-PKG composite site to identify which of these compounds showed inhibition. PDE5A was incubated with three compounds G, H and I and added to 2fg-PKG. Decreased polarization was observed for compound I incubated PDE5A, suggesting that this did not inhibit PDE5. FP values for PDE5A treated with compounds G and H did not change over the time-course of the experiment, indicating that these two were potential PDE5A inhibitors.

Current methods employed for the screening and detection of PDE5 inhibitors and their analogues as adulterants in herbal products include HPLC-UV, MS-based methods (e.g. LC-MS, GC-MS), vibrational spectroscopic methods (e.g. IR, Raman spectroscopy) and NMR spectroscopy. However, such targeted, structure-based techniques possess a major limitation of the need for prior knowledge of the chemical structure. Hence, they have limited usefulness in the identification of new analogues with different chemical structures from that of known PDE5 inhibitors or when reference standards are lacking. In contrast, the developed FP assay is an untargeted activity-based bioassay where detection of any adulterants is based on their pharmacological mechanism (i.e. PDE5 inhibition). Thus, its utility as a qualitative screening assay for the detection of PDE5 inhibitor analogues in natural products was investigated. As can be seen from Figure 3(iv), addition of PDE5 pretreated with C5-4 alone resulted in a drop in FP values similar to that of active PDE5. This indicates that C5-4 is unable to inhibit PDE5 and it is in line with previous results. On the other hand, addition of C5-4 spiked with the sildenafil analogues led to a slight drop in FP values that were between that of active and inactive PDE5. This suggests that the extract spiked with sildenafil analogue partially inhibits PDE5. Therefore, the results indicate that the detection capability of the developed FP assay towards the sildenafil analogues is retained even in a complex matrix such as a semi-purified fraction of a plant extract and may potentially be applied for the screening of herbal products adulterated with PDE5 inhibitors in future.

Therefore, using PDE5A-PKG complex as a probe in fluorescence polarization assay, we were able to screen extracts that inhibit/affect the cGMP kinase and cGMP hydrolyzing PDEs. Using the composite site as the target and the assay with a short incubation step, we are able to rapidly distinguish between an inhibitor and non-binding molecule. We believe that this approach has enormous implications in identifying novel compounds that bind to both kinase and phosphodiesterase, and be used as high-throughput screening procedure.

### 2.3 Ranking inhibitors targeting the specific PDE-PK complexes

Using various extraction procedures, a range of mixtures were isolated from the two species of medicinal plants examined. The potential PDE inhibition activity of these mixtures and fractions was tested and quantified using a commercial luminescence-based PDE-Glo™ phosphodiesterase assay and compared with results obtained using fluorescence-based PDE-PK composite site. Initially, the PDE inhibition of all extracts was determined by luminescence assays using PDE5 alone. After 15 min of incubation of PDE5 with each extract and cGMP was added, an additional 30 min of the luminescence values were detected according the manufacturer's prescribed protocol. Different extracts showed varying degree of inhibition of PDE5, wherein certain extracts having no (extracts B), moderate (extracts D and E) or high (extracts A, E, and F) potency, as compared to the known inhibitor Sildenafil. From these, only certain plant extracts were considered for further analyses to compare the results with fluorescence polarization experiments. Extracts from both plants prepared from a wide range of solvents (see methods) showed significant PDE5 inhibition ( $p < 0.05$ ; compared to control), indicating the presence of multiple active constituents with varying polarities.

PDE-Glo™ phosphodiesterase assay was carried out for extracts A, B, C, D, E, and Sildenafil (for PDE5) and PF-04957325 (for PDE8) were used as positive controls. Results obtained from PDE-Glo™ phosphodiesterase assay revealed that extracts A, C and E significantly inhibited PDE5 while extract B did not inhibit PDE5 (Fig. 4 ii). On the other hand, extracts A and B inhibited PDE8 only moderately, while extracts C and E showed high degree of inhibition (Fig. 4 i). The potency of these extracts as potential PDE5 inhibitors in decreasing order is C>A>E, where extract C showed near complete inhibition while extract E had the least effects. However, the potency of PDE8 inhibition was observed as E>D>B>A with extract E blocking the PDE8 active site comparable to PF-04957325. Notably, order of inhibition of PDE5 (Fig. 4 ii) and PDE8 (Fig. 4 i) by the plant extracts is different. Based on this preliminary screening, crude extract C was selected for further fractionation and evaluation of their PDE inhibitory potential.

PDE-Glo™ phosphodiesterase assay was performed for fractions C2, C3 and C5 to estimate their inhibition potency. Fractions C2 and C3 showed low inhibition, while fraction C5 showed PDE5A inhibition comparable to that of Sildenafil (Fig. 4 ii). Subsequently, semi-quantitation of the sub-fraction C5-3 and the compounds G, H, and I identified from C5-3 was also carried out. PDE-Glo™ assay results were similar to those observed for FP, with compounds G and H inhibiting PDE5A to ~90% while PDE5A remained active in the presence of compound I.

Subsequently, we qualitatively compared the results from luminescence assay versus the fluorescence polarization assay, as shown in Fig. 4. While the results were consistent for PDE5 with similar % inhibition and extract C being the most potent plant extract; the comparison for PDE8 were different. Incubation of PDE8 with extract B for 15 min showed no inhibition by PDE-Glo™ results (Fig. 4 i), but the fluorescence polarization results clearly show a time-dependent inhibition (Fig. 2 i, orange triangles). At earlier times (20-40 min), the FP values were low, indicating PDE8 inhibition, but at later times (60-80 min) the FP values increased to values similar to active PDE8. The PDE-Glo™ phosphodiesterase assay (i) relies on stopping the PDE catalysis using IBMX and (ii) takes ~3 h in total, and thus this assay introduces a bias. Therefore, extract B showed no inhibition in the luminescence assay, which is similar to FP values observed during later times. Therefore, targeting the PDE-PK composite site by fluorescence polarization approach yields more information and insights into the stability of the compound, about the dwell time of the inhibitor in the catalytic site, and on the effects of other nucleotide-like molecules in the mixture.

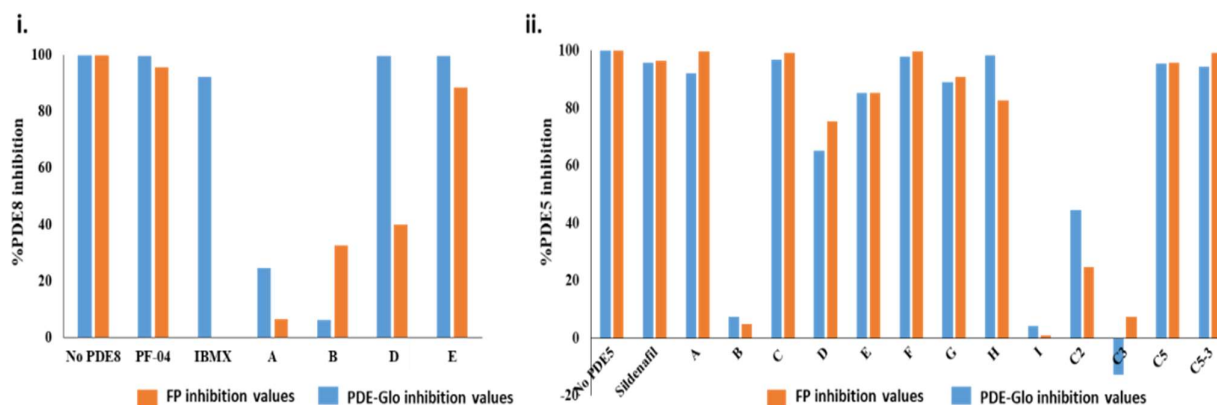


Figure 4: Comparison of PDE-Glo™ and FP assay results for PDE inhibition.

The results for fluorescence polarization (orange) and luminescence-based PDE-Glo™ (blue) for PDE8 (i) and PDE5 (ii) are compared and depicted. No PDE8 or PDE5 were used as negative controls for maximal signal (100%); PF-04957325 (99.8%) and Sildenafil (98.5%) inhibitors were used as positive controls for highest inhibition. PDE8 was incubated with extract A, B, D and E. Disparity is seen between the two assays for extracts A and B. (ii) Effect of extracts A-F, compounds G, H and I and fractions from extract C on PDE5

inhibition is compared for the two assays. Inhibition values for FP and PDE-Glo™ assays were calculated as described in methods and the error bars were too small to be visible. The values are tabulated in Supplementary information Table S1.

### 3. Discussion

Cyclic nucleotide signaling integrates multiple signaling and metabolic events in cells and hence its fine-tuned spatio-temporal regulation is critical. With advanced techniques the phosphodiesterases are now recognized to act in conjunction with other macromolecules, notably the cNMP-effector proteins in signalosome assemblies. This increased focus on signalosome assemblies warrants examining PDEs as enzymes complexed with cNMP-target proteins, rather than as free PDEs in solution. Here, in this study, we report and discuss the importance of targeting the composite PDE-PK active sites for identifying specific inhibitors. We earlier showed that the PDE-PK composite site is catalytically highly efficient and the preferred route of cyclic nucleotide turnover [14, 18]. Many research studies have shown that inhibition of phosphodiesterases leads to increased PKA activity, but the mechanism of activation is yet not described [8, 30]. The PDE-PK composite site is the 'missing link' and provides a rationale for activation of PKA when PDEs are inhibited. This is possibly because PDEs exist in complexes with cyclic nucleotide receptors (PKAR), and the inhibited 'composite sites' prevent or delay cAMP signal termination. Through an extensive analysis of different substrates and their concentrations, we propose PDE-PK complex as the probe to determine inhibition potency. We propose a time-resolved fluorescence polarization assay that offers a dual read-out of binding of small molecules by dissociation/displacement and the degree of enzyme activity.

The phosphate binding sites of protein kinases (PKA/PKG) and the phosphate hydrolysis sites of phosphodiesterases (PDE8/PDE5) couple to form a receptor-hydrolase complex. This coupled active site bound to fluorescent analog of cNMP\*, i.e., the 'R<sub>cNMP</sub>\*-PDE probe' (Fig. 5, green box) serves as an excellent method to determine cAMP/cGMP hydrolysis and their binding kinetics. This was tested with different concentrations of substrate and observed that PDE-PK composite site hydrolyzed all substrate molecules followed by reassociation as R<sub>cNMP</sub>\*-PDE probe. As PDE hydrolyzes 2fluo-cAMP to 2fluo-AMP and the PDE-PK complexes bind fluorescent analog of the product as well, offering itself as a robust probe to monitor the active, inactive and end-state complexes. Moreover, small molecules which activate the enzymatic activity (Fig. 5, blue box) or are weak competitive inhibitors with delayed response can also be successfully screened using this time-resolved assay. The power of this assay is to qualify a potential biomolecule as a strong or weak ligand with simultaneous measurements of the dwell time, competitive displacement of substrate/product, and the level of inhibition (Fig. 5, red box). Besides, comparing known and novel inhibitors, semi-quantitative analysis of efficacy (IC<sub>50</sub>) and duration of inhibition can be inferred as well. For instance, extract E displaces 2fluo-cAMP instantly from RegA-R<sub>D</sub> complex, faster than cAMP, indicating that the affinity of compounds in extract E is higher than cAMP (< 2 nM).

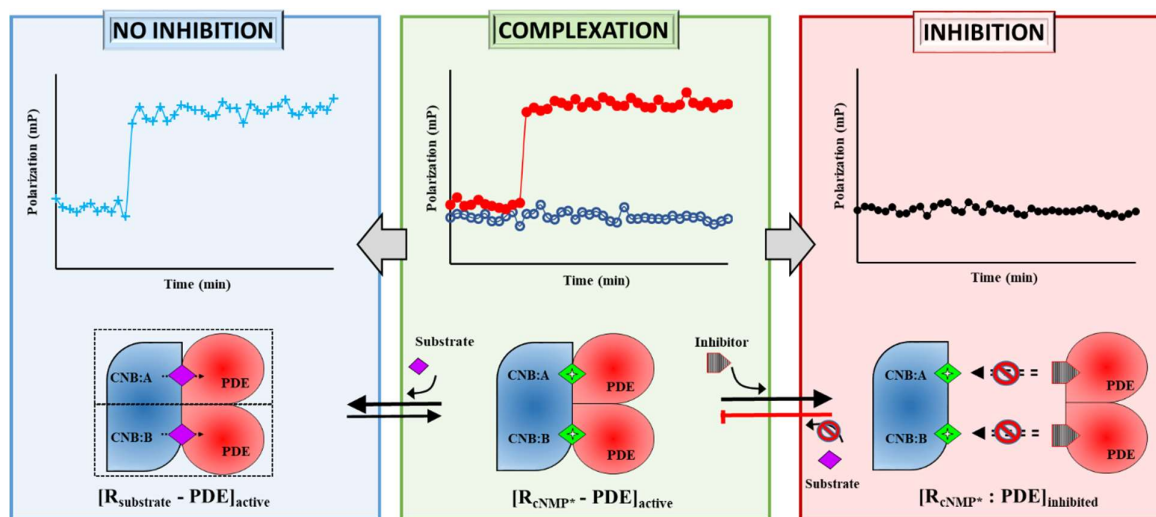


Figure 5: PDE-PK composite site as the new target for drug discovery.

(Centre panel) The cyclic nucleotide binding domains (CNB) of protein kinase, the 'receptor' (R) and catalytic site of phosphodiesterase ('PDE', red) coupled in the presence of fluorescent analog of cyclic nucleotide (green diamond, cNMP\*) to form an  $[R_{cNMP^*}\text{-PDE}]$  active complex (red circles plot). (Left pathway) The stability and activity of the complex was tested by addition of cyclic nucleotide substrates (purple) resulted in competitive displacement of the fluorescent analogs and leading to a catalytic active  $[R_{\text{substrate}}\text{-PDE}]$  complex. The displaced fluorescent analog of cAMP reassociates with PDE-PK complex resulting in increased FP (cyan plus plot). (Right pathway) Inhibitors (grey) bind to and block the active site and prevent composite site formation yielding an  $[R_{cNMP^*}\text{-PDE}]$  inhibited complex as observed by no changes in FP (black circles plot).

This  $R_{cNMP^*}\text{-PDE}$  probe uses changes in fluorescence polarization intensities measured over the course of the reaction. Fluorescence polarization has been widely applied as a high-throughput screen to identify inhibitors from a large library [37]. Traditionally, PDEs have been targeted alone by fluorescence assays [38], or using radioactive-labeled cyclic nucleotides such as  $[^3\text{H}]$ ,  $[^{14}\text{C}]$  or  $[^{32}\text{P}]$  cAMP/cGMP as substrates [38-40], cell-based assays [41]. Alternatively, luciferase-luciferin coupled detection of ATP levels in cAMP signaling pathway has been developed to quantify the PDE activity by luminescence. However, these assays for screening lead compounds require multiple steps of incubation, dose-dependent and PDE-concentration dependent repetitive experiments, including cell-culture methods.

One of the most important concerns of these assays is non-specificity of the small molecules targeting all PDEs, with little or no information on isoform-specific inhibition. This warrants a need for a robust assay that determines specific PDE inhibition, provides insights into their efficacy and allows rapid screening and identification of potential novel small molecules. Recently, a NMR-based phosphodiesterase assay was developed based on quantification of the product 5'-AMP [42]. This is limited in requiring NMR expertise and used free PDEs as targets. To this end, with our approach using PDE-PK probe as the target, we were able to screen various test molecules rapidly, without any optimization requirements. One major advantage of this approach is that it can be customized for specific PDE, present as a signalosome complex.

Although the luminescence assay results provide an overall view of the PDE inhibition by the various extracts, they do not provide detailed insights into the mechanism of action or whether the phytoconstituents bind directly to PDE active site or not. On the other hand, with the fluorescence polarization assay using PDE-PK probe as the target, we uncovered more details on inhibitory effects of these extracts (Table 1).



**Table 1:** Summary of the % inhibition of extracts obtained from fluorescence polarization assay results using specific PDE-PK pair as the probe.

Extract	% Inhibition			Characteristics
	PDE8-PKAR	PDE5-PKG	RegA-R <sub>D</sub>	
A	6.5%	99.8%	61.1%	Potent inhibition of cGMP - selective PDEs
B	32.6%	4.9%	75.0%	Partial inhibition of cAMP - specific PDEs
D	40.1%	75.5%	48.6%	Partial inhibition of general PDEs
E	88.6%	85.3%	99.5%	Potent inhibition of general PDEs
F	30%	98%	50%	Inhibition of cGMP-selective PDE sites
Sildenafil	-*	96.5%	n.d*	Known PDE5-specific inhibitor
PF-04957325	95.6%	-*	n.d*	Known PDE8-specific inhibitor

\*n.d.: not determined

Firstly, our results clearly show that certain extracts have natural products that are more potent than others, and also report on their time-dependent inhibition. While other assays give an end-point result, our approach gives a real-time readout of the effect of inhibitors on the active sites. In particular, extract B does not inhibit PDE5, but shows high PDE8 inhibition at early times and decreased inhibition at later stages (60-80 min, similar to luminescence results). Consequently, limitations of existing PDE assays would have deemed extract B as non-inhibitory, while our FP assay clearly shows that this extract may inhibit PDE. Second, using two different composite sites, we were able to distinguish broad-specific from isoform-specific inhibitors (Table 1). Our results show that extracts A or E are broad-specificity inhibitors, extract B, compounds G, H are isoform-specific inhibitors. Third, the PDE-PK composite probe allowed us to distinguish between different cyclic nucleotide binding sites. This specificity of the extracts to either cAMP or cGMP is well-highlighted by the effects of plant extract G. Luminescence assay qualifies it as PDE inhibitor, but fluorescence polarization assay specifies it as a cGMP-specific inhibitor. This site-specificity of the assay is useful in screening small molecules for PDEs that show dual-specificity (PDEs 1, 2, 3, 10, 11) and also provide insights into isoform-selectivity of the identified compound. Together these results emphasize the importance of targeting PDEs as complex active sites, rather than free PDEs.

In literature, free phosphodiesterases have been the sole targets for drug design and small molecule therapeutics. In many cases, this approach has led to discovery of inhibitors with unintended side effects. A vast majority of approved compounds interact at the conserved catalytic sites and result in cross-reactivity with ‘off-target’ proteins. With over 100 PDEs present, there is therefore an urgent need of identifying novel isoform-specific inhibitors. Our proposed assay is based on competitive binding between substrate and test molecule to determine the catalytic activity. Here, we show that PDE-PK complex acts a sensitive and rapid fluorescence polarization probe and is able to provide insights into lead compounds or identify natural products as potential PDE-PK inhibitors.

4. Materials and Methods

4.1 Materials

Ultra-competent *Escherichia coli* BL-21 (DE3) bacterial strains used for protein expression were obtained from Life Technologies (Carlsbad, CA). TALON® cobalt resin for affinity purification was from ClonTech (Mountain View, CA) while BioGel HTP hydroxyapatite beads were from BioRad laboratories (Hercules, CA). Analytical grade organic solvents (hexane, dichloromethane, ethyl acetate, ethanol, methanol) and HPLC-grade methanol were purchased from Tedia (Fairfield, OH, USA). MilliQ (Merck, Darmstadt, Germany) water was used. PDE-Glo™ Phosphodiesterase Assay kit was purchased from

Promega Corporation (Madison, WI, USA). LC/MS grade acetonitrile, methanol and water were from Fisher Scientific (Waltham, MA); and trifluoro acetic acid (TFA), sequence analysis grade from Fluka BioChemika (Buchs, Switzerland). Deuterium oxide was from Cambridge Isotope Laboratories (Tewksbury, MA). All other reagents and chemicals were research grade or higher from Sigma-Aldrich (St. Louis, MO).

Active recombinant human PDE5A was from SignalChem Pharmaceuticals Inc. (Richmond, Canada) and active recombinant human cGMP-dependent Protein Kinase Type 1 (PKG1) was obtained from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was procured from Sigma-Aldrich (St. Louis, MO, USA) while phosphate buffer saline (PBS) was from Vivantis Inc. (Oceanside, CA, USA). Sildenafil citrate was acquired from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) while 3-isobutyl-1-methylxanthine (IBMX) was from Sigma-Aldrich (St. Louis, MO, USA). PF-04957325 was from Med-ChemExpress (Singapore). Fluorescent analogs 2'-fluoro-AHC-cGMP (2'- (6- [fluoresceinyl] aminoethylcarbamoyl) guanosine- 3', 5' - cyclic monophosphate) and 2'-fluoro-AHC-cAMP (2'- (6- [fluoresceinyl] aminoethylcarbamoyl) adenosine- 3', 5' - cyclic monophosphate), henceforth referred to as "2fluoro-cGMP" and "2fluoro-cAMP" respectively, were obtained from Biolog Life Science Institute (Bremen, Germany).

## 4.2 Methods

### 4.2.1 Expression and purification of proteins

Recombinant proteins used in this study were PKAR (regulatory subunit of bovine cAMP dependent protein kinase PKA), R<sub>0</sub> (regulatory subunit of *Dictyostelium discoideum* cAMP dependent protein kinase PKA), PDE8c (catalytic domain of human PDE8A1), and RegA (type 2 phosphodiesterase from *Dictyostelium discoideum*). Each of these proteins were recombinantly expressed in *E. coli* competent cells and purified as described previously [6, 18, 21]. The purity (>95%) and homogeneity of each of the proteins were confirmed using size-exclusion chromatography and denaturing acrylamide gel electrophoresis. cGMP-dependent protein kinase (PKG) and cGMP-specific phosphodiesterase (PDE5) were obtained commercially.

### 4.2.2 Preparation of plant extracts

Two medicinal plant species [43-45] were collected locally and their voucher specimens were kept at the Herbarium of Department of Pharmacy, National University of Singapore. Extraction using different solvents (e.g. ethanol, methanol, water) and extraction methods (maceration, ultrasonication, Soxhlet extraction) yielded different plant extracts and extracts A to F were used in this study. Extracts A to C were obtained from *Swietenia macrophylla* King seed kernels (Plant 1) and D-F from *Vitex trifolia* L. var. *trifolia* leaves (Plant 2) (Supplementary Figure S1). The extracts were subsequently dried *in vacuo* and separately dissolved in an appropriate volume of 4% v/v DMSO in PBS to obtain a concentration of 5 mg/ml each.

Normal phase adsorption chromatography was performed on extract C using Silica gel (Si-gel) 60 (0.063-0.200 mm, Merck, Germany). Stepwise elution was performed using n-hexane-ethyl acetate (EA)-methanol (MeOH), starting with n-hexane:EA (1:0 @ 0:1, v/v) followed by EA:MeOH (1:0 @ 0:9, v/v) as a gradient elution system. Based on their thin layer chromatography profiles, similar fractions were pooled together to afford fractions C1 to C6. Fraction C5 was subsequently selected (based on its PDE5 inhibitory activity) for further fractionation to yield subfractions C5-1 to C5-4. Compounds G and H were identified from extract C via Gas Chromatography-Mass Spectrometry (GC-MS). Column chromatography of extract C also led to the isolation of compound I.

### 4.2.3 Fluorescence Polarization

Fluorescence polarization (FP) assays were performed using PDE-hydrolyzable fluorescent analogs 2fluoro-cAMP and 2fluoro-cGMP. Initially, PKAR, R<sub>0</sub> and PKG were incubated for 18 h with a molar excess of these fluorescent ligands to saturate their CNB sites.

Unbound ligands were removed by dialysis and size-exclusion chromatography. Each of these ligands bind their respective receptors with very high affinity and do not dissociate easily [22, 46, 47]. These proteins bound to analogs are henceforth referred to as '2fc-PKAR', '2fc-Rb', and '2fg-PKG' for the respective proteins bound to 2fluo-cAMP or 2fluo-cGMP, respectively. FP assays were performed in 96-well flat bottom opaque non-binding black plates and measurements were recorded using Synergy 4 multi-detection microplate reader (BioTek, Winooski, VT). For all ligands, excitation wavelength  $\lambda_{ex}$  = 485 nm and emission wavelength  $\lambda_{em}$  = 524 nm were used with a bandwidth of 20 nm and instrument G-factor being 0.87.

#### 4.2.3.1 Designing the PDE-PK complex as target

In the first set of experiments, FP of 2fc-PKAR (0.5  $\mu$ M), 2fg-PKG (0.1  $\mu$ M), and 2fc-Rb (1  $\mu$ M) were recorded for initial 20 min. Then:

- free PDE8c (1  $\mu$ M) and PDE8c saturated with cAMP (250  $\mu$ M), IBMX (500  $\mu$ M) or PF-04 (10  $\mu$ M) were added separately to 2fc-PKAR at t = 20 min.
- to 2fg-PKG, cGMP (100  $\mu$ M) and PDE5 (0.2  $\mu$ M) with or without sildenafil (1  $\mu$ M) and cGMP were added independently and their FP values were measured for additional 60 min.
- FP values of 2fc-Rb (1  $\mu$ M) and 2fc-Rb with RegA (2  $\mu$ M) were recorded for 20 min, followed by addition of cAMP (250  $\mu$ M), cGMP (250  $\mu$ M) or IBMX (500  $\mu$ M) to preformed 2fc-Rb-RegA complex and FP was measured for total time of 100 min.

#### 4.2.3.2 Testing PDE-PK complex with known inhibitors

In the second set of experiments, IBMX (500  $\mu$ M) or PF-04957325 (1  $\mu$ M) saturated PDE8c was added to 2fc-PKAR at time t = 20 min and the FP was recorded for further 60 min. PDE5A incubated with cGMP (100  $\mu$ M) or Sildenafil (1  $\mu$ M) was added to 2fg-PKG after 30 min of initial baseline FP measurements. For RegA-Rb pair, two different set ups were tested. First, cAMP (250  $\mu$ M) was added to 2fc-Rb initially (0 min) followed by adding RegA at 20 min; and second, IBMX (250  $\mu$ M) was added to 2fc-Rb-RegA complex at 20 min. FP was then measured for total time of 100 min.

#### 4.2.3.3 Targeting PDE-PK complexes with plant extracts

In a third experimental set-up, PDE8c (1  $\mu$ M) was incubated with extracts A-F for 15 min and added to 2fc-PKAR at 20 min. To preformed 2fc-Rb-RegA complex, 5  $\mu$ l of extracts A-F (1 mg/ml) were added at 20 min and the FP was recorded for total time of 80 min.

PDE5 (1  $\mu$ M) was incubated with 5  $\mu$ L of plant extracts A-F, C2, C3, C5, C5-3, C5-4 and compounds G, H and I (1 mg/ml each) for 15 min and added to 2fg-PKG in separate wells and the FP was measured for additional 60 min. In addition, PDE5 was also incubated with sub-fraction C5-4 was spiked with 100 nM Sildenafil for 15 min, and then added to 2fg-PKG. Consecutive FP measurements were taken at time intervals of 2 min. All experiments were carried out in duplicates and averages from two independent measurements were then plotted.

FP based inhibition of PDE by the extracts were calculated as ratios of differences in polarization of active and inhibited PDE to differences in polarization of PDE-bound and free Protein Kinases (PK), as per following equation:

$$\% \text{ PDE inhibition (FP)} = \left( 1 - \frac{\text{FP}(\text{sample}) - \text{FP}(\text{PDE})}{\text{FP}(\text{PK}) - \text{FP}(\text{PDE})} \right) \times 100\%$$

where FP(PDE) is the average FP value of active PDE bound to PK, FP(sample) is the average FP of PDE incubated with extracts/compounds, and FP(PK) is the average FP value of Protein Kinase without PDE.

#### 4.2.4 PDE-Glo™ Phosphodiesterase Assay

PDE inhibitory activities of the plant extracts were also investigated using the PDE-Glo™ phosphodiesterase assay as per manufacturer's instructions. In brief, 5 µL of plant extract/known inhibitor was incubated at room temperature with 7.5 µL of the active recombinant human PDE5A (0.3 ng/µL) or PDE8c (0.3 ng/µL) for 15 min. The final concentration of plant extracts in each well was 1 mg/mL while that of Sildenafil was 1 µM, IBMX at 100 µM, and PF-04957325 at 1 µM. PDE reaction was then initiated by adding 12.5 µL of 20 µM cGMP or 2 µM cAMP (1 mM for PDE8c) to each reaction and incubating the mixture at room temperature for 1 h. The reactions were then terminated and the luminescence was detected according to manufacturer's instructions.

PDE-Glo™ phosphodiesterase assay measures PDE mediated catalysis of cyclic nucleotides indirectly by conversion of ATP levels by luciferin-luciferase system. The assays were performed in 96-well opaque white plates from SPL Life Sciences (Korea) and luminescence was measured using Promega GloMax®-Multi Detection System (Madison, WI, USA). The luminescent signal produced is directly proportional to the remaining ATP levels and hence, directly correlates with PDE activity. Thus, greater the inhibition potency of the plant extracts/fractions, the lower the luminescent signal detected. PDE aliquoted in 4% DMSO in PBS was used as negative control, while test well containing only cGMP (i.e. no inhibitor and no PDE) was also included as PDE activity control (akin to 100% PDE inhibition). PDE inhibition of the plant extracts/known inhibitor was calculated using the following equation:

$$\% \text{ PDE inhibition (PDE}_{\text{Glo}}) = 1 - \frac{[\text{RLU}(\text{sample})]}{\text{RLU}(\text{negative control})} \times 100\%$$

where RLU (sample) is the average relative luminescence units of the specific sample/plant extract, RLU (negative control) is the average relative luminescence units detected for the negative control. The results were generated from at least three independent experiments and each experiment performed in duplicates.

## 5. Conclusions

Current repertoire of available assays lack the ability to profile the isoform-specificity, efficacy and potency of the inhibitors as these are designed to target exclusively free PDEs. Free PDEs passively hydrolyze bulk cyclic nucleotides in cytoplasm and represent a subset of the total pools of intracellular PDEs. Our assay characterizing PDE complexes in signalosomes paves the way for identifying PDE inhibitors with greater specificity and lower cross reactivity. The PDE-PK complex as a pharmacological target offers a new route for accelerating discovery of new class of inhibitors with greater implications in understanding the precise regulation of compartmentalized cyclic nucleotide signaling.

**Supplementary Materials:** Figure S1: Activity-guided fractionation of plant extracts, Table S1: Supplementary Table S1: Inhibition values for various extracts and fractions inhibiting PDE8 and PDE5, as determined by fluorescence polarization (FP) and PDE-Glo™ assays.

**Author Contributions:** Conceptualization, N.K.T, G.S.A, and K.H.L.; methodology, N.K.T and S.J.E.V.; formal analysis, N.K.T, S.J.E.V.; investigation, K.H.L. and G.S.A; resources, N.K.T., K.H.L. G.S.A; data curation, N.K.T.; writing—original draft preparation, N.K.T, and S.J.E.V.; writing—review and editing, N.K.T, S.J.E.V., G.S.A, and K.H.L.; visualization, N.K.T. and S.J.E.V; supervision, K.H.L and G.S.A; project administration, K.H.L and G.S.A; funding acquisition, K.H.L., G.S.A. All authors have read and agreed to the published version of the manuscript.

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