The Characterization of the Interaction Between Phosphodiesterase 3A and Compound 1B, a Novel Selective Cytotoxic Agent

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The Characterization of the Interaction Between Phosphodiesterase 3A and Compound 1B, a Novel Selective Cytotoxic Agent

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Biological Chemistry
Wellesley College
Spring 2014

Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

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ABSTRACT

The field of cancer therapeutics is moving towards the development of targeted cancer therapies – therapies that selectively kill cancer cells by targeting genomic features crucial to tumor survival. Compound 1B is a novel cytotoxic agent that is selectively toxic to 4% of cancer cell lines. After sensitive cell lines were treated with the compound for 48 hours, they exhibited apoptotic cell death with a half maximal effective concentration (EC50) in the low nanomolar range. This study represents the initial steps in understanding the mechanism of selective toxicity of Compound 1B using a two pronged approach that includes predicting the binding of Compound 1B to its proposed target phosphodiesterase 3A (PDE3A) using available structural information, as well as investigating experimentally its effect on the enzymatic activity PDE3A. Our findings suggest that Compound 1B binds to the catalytic domain of PDE3A, and therefore acts as a competitive inhibitor of cAMP hydrolysis. In order to validate that PDE3A was interacting with Compound 1B and to measure the effect of the compound on the kinetics of PDE3A, an enzymatic activity assay was developed. Our results showed that Compound 1B bound to the catalytic site of PDE3A, inhibiting its cAMP hydrolysis function in a similar manner to known competitive PDE3A inhibitors. Additionally, Compound 1B affected the kinetics of PDE3A in a similar manner to competitive inhibitors Zardaverine (lethal to Compound 1B-sensitive cell lines) and Cilostazol (non-lethal to Compound 1B-sensitive cell lines). The interactions between Compound 1B and PDE3A were not unique to lethal compounds because Compound 1B interacted with PDE3A just as non-lethal Cilostazol did. Therefore, the ability of Compound 1B to inhibit cAMP hydrolysis was not sufficient to explain the selective apoptosis observed in sensitive cell lines. In the Hela cell line, Compound 1B competed with non-lethal PDE3A competitive inhibitors, as treatment with these inhibitors rescued the lethal effect of Compound 1B. This result confirmed that the binding of Compound 1B to PDE3A, as seen in vitro, occurred in cells and was linked to the cytotoxic phenotype of Compound 1B. Compound 1B behaved as a competitive inhibitor of PDE3A in crystallized models, in vitro experimentation, and in the Hela cell line. Elucidating the functional effect of this competitive binding in terms of the connection to the selective toxicity of Compound 1B, is essential for its potential as a treatment for human cancer.
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ACKNOWLEDGEMENTS

I would like to thank Luc de Waal and Dr. Matthew Meyerson for giving me the most fulfilling research experience at the Broad Institute of MIT and Harvard for the past two years. You have been the most incredible mentors. You have taught me a new way of thinking critically and have never ceased to challenge me scientifically. Thank you for always believing in me when I needed to retain confidence. Your advice has been truly invaluable; I could not be more appreciative that you always pointed me in the right direction for any additional resources I may have needed. Your enthusiasm for science has instilled in me a deep appreciation for the process. It was such an honor to work with you, and I know I can never thank you enough. I would like to thank all other members of the Meyerson Laboratory for their constant encouragement on my thesis project and as a developing scientist.

I am grateful to my professors at Wellesley College for their unwavering support. Professor Vardar-Ulu, you made it possible for me to enrich my Wellesley College experience with my off-campus research for four semesters. Thank you for your time, expertise, patience, and guidance. Professor David Haines, Professor Louise Darling, and Professor Stacie Goddard, thank you for serving as members of my thesis committee. Thank you for enriching our conversations with your unique scientific perspectives and reminding me to maintain a broad outlook on my project. I truly appreciate all the time and encouragement that you generously gave me.

Thank you Professor Elmore for always answering my endless scientific and procedural questions. You have provided such structure to this experience. Your constant presence throughout the process was incredibly reassuring. I couldn’t have pulled my thesis together without you.

I am grateful to Professor Klepac-Ceraj for constantly checking in with me and taking such an interest in my thesis experience. You have not only offered support in numerous way, but also enriched many aspects of this project with your scientific expertise.

And finally, thank you to my loving family and friends for creating the most fabulous support network. None of my dreams would be achievable without all of you.

The extent of my research would not have been possible without the generous gift from the Jerome A. Schiff Charitable Trust.
INTRODUCTION

Cancer is responsible for 7.6 million deaths worldwide and is projected to cause 585,720 deaths in 2014 in the United States alone\(^1\). Current treatment for cancer focuses on chemotherapeutic agents that kill rapidly dividing cells, a characteristic of cancer cells, but also a characteristic of cells that rapidly divide under normal circumstances such as cells of the bone marrow, digestive tract lining and hair follicles\(^2\). The effectiveness of chemotherapy is substantially reduced by the side effects in normal tissues, such as kidney toxicity associated with cisplatin treatment, a common anti-cancer drug\(^3\). Because of this toxicity, the therapeutic window of chemotherapy is narrow, and the dose for eradicating all of the cancer cells cannot usually be administered\(^3\). Since all cancer cells are not typically killed during chemotherapy, small numbers of resistant populations of the cancerous cells can survive and will not be easily killed by subsequent treatments of traditional chemotherapy.

Due to these drawbacks of traditional chemotherapy, modern cancer therapy presents a more focused approach for eradicating the cancer cells. It centers on targeting cancer-specific genomic features deregulated in only cancer cells and not healthy cells. The first targeted cancer therapy was designed to inhibit estrogen from binding to estrogen receptors. Because blocking estrogen from binding to the estrogen receptor had been shown to prevent breast cancer cell growth and proliferation, the FDA approved several drugs such as tamoxifen and toremifene\(^4\), which serve to specifically prevent estrogen from binding to the estrogen receptor.
Often, as in the case of the discovery of tamoxifen, the desired molecular target is identified, and molecules are developed to specifically interact with the target. The small molecule investigated in this study, Compound 1B, is selectively toxic to few cancer cell lines, but its cellular target is unknown. This study represents the initial steps in understanding the mechanism of selective toxicity of Compound 1B, a step crucial for the therapeutic application of the compound.

**Dose-Dependent Cytotoxic Effect of Compound 1B on Cancer Cell Lines**

Compound 1B originated from a small-molecule screen intended to find a mutant TP53 synthetic lethal compound, a compound that acts in combination with a mutation inherent in cancer cells to cause lethality (Heidi Greulich and Luc de Waal, Unpublished Data, 2012). TP53 is the most frequently mutated gene across all cancers at rates of 95% in ovarian cancer, 84% in lung squamous cell carcinoma, and 51% in lung adenocarcinoma. Because TP53 regulates the cell cycle and conserves stability in the genome, mutations will often lead to unregulated cell division. Compound 1B was selected from the NIH’s Molecular Libraries Probe Production Center (MLPCN) pilot screen of 2,000 compounds because it was selectively toxic to a TP53 mutant cell line (H1734), but was not toxic to a TP53 wildtype cell line (A549), two non-isogenic cell lines that shared other major cancer drivers, KRAS and STK11.

In order to gain an understanding of the genomic features that accounted for this sensitivity, the panel of cell lines treated with Compound 1B was extended. Compound 1B was found to be selectively toxic to 4% of the 766 cell lines in the Cancer Cell Line Encyclopedia (CCLE). Representative cell lines, Hela (cervical cancer), A549, H1563
and H2122 (lung cancer epithelial cell lines) and MCF7 and PC3 (breast cancer cell lines) were incubated for 48 hours with Compound 1B (Figure 1). Hela, H2122 and H1563 cells showed a dose-dependent decrease in viability as concentration of Compound 1B increased from 0.1 pM to 300 µM. The A549, MCF7, and PC3 cell lines however did not show a decrease in viability after treatment with Compound 1B until extremely high doses of compound at greater than 100 µM were administered. Notably, there is a large variation among the sensitivity of the cell lines as seen by the 10,000 fold change in EC50 values, a measure of the concentration of Compound 1B required to cause death in 50% of the cells.

![Graph showing dose-dependent cytotoxic effect of Compound 1B on viability.](image)

**Figure 1: Dose-Dependent Cytotoxic Effect of Compound 1B on the Viability of Cancer Cell Lines.** Cancer cell lines, Hela, A549, MCF7, PC3, H1563, and H2122, were treated with doses of Compound 1B from 0.1 pM to 300 µM for 48 hours. Cell viability was assessed using the Cell-Titer Glo assay (Promega). (Luc de Waal, Unpublished Data, 2012).
PDE3A, the Single Gene Correlate for Sensitivity to Compound 1B

The cell line sensitivity data was used to predict a single gene that correlates with sensitivity to Compound 1B. The Pearson product-moment correlation was used to measure the strength of the linear association between the two variables, sensitivity to Compound 1B and gene expression level. Sensitivity was measured as area under the dose-response curve (AUC) from the upper plateau at 100% viability to 0% viability; regardless of whether a cell line reached this 0% viability, the total area to this point was still calculated. As seen by the curves in Figure 1, a sensitive cell line would have a low AUC, and a nonsensitive cell line would have a high AUC. PDE3A was the single significant gene correlate based on this input of the sensitive and the non-sensitive cell line mRNA expression levels (Figure 2). PDE3A expression correlated with a low AUC and high sensitivity. Figure 2B shows the distribution of cell lines in terms of their lineage, sensitivity to Compound 1B, and PDE3A expression. Although skin cancer cell lines are overrepresented in the sensitive cell lines with high PDE3A mRNA expression, there is no correlation between all three variables. Because high PDE3A expression was hypothesized to correlate with cell line sensitivity to Compound 1B, we sought to determine if PDE3A is a target of Compound 1B and if this interaction is responsible for the selective toxicity of the cell lines.
Figure 2: PDE3A Predicted as Single Gene Correlate that Distinguished Compound 1B Sensitive Cell Lines from Nonsensitive Cell Lines. A. Pearson product-moment correlation found the strongest linear relationship between mRNA expression of PDE3A and cell line sensitivity to Compound 1B. B. Sensitivity to Compound 1B from expression and sensitivity data of the 766 cell lines from the CCLE plotted as AUC (area under the curve of the viability assay from 100% viability to 0% viability) versus mRNA expression level of PDE3A transcript (Matt Reese, Unpublished Data, 2013).

An Overview of PDE3A Biology

PDE3 is one of 11 families of phosphodiesterases which all hydrolyze cyclic nucleotides but differ in their structure, substrate affinity, and regulation. The catalytic site for this hydrolysis is composed of about 270 amino acids at the C-terminal end of the protein and is highly conserved among the 11 families of phosphodiesterases (24-51% conservation). The domain is primarily composed of alpha helices and is characterized by two divalent atoms, usually Mg$^{2+}$ or Zn$^{2+}$, crucial for its catalytic function. Within the PDE3 family there are two genes, PDE3A and PDE3B, which are structurally unique from the other phosphodiesterase families because they possess a 44 amino-acid insert in the catalytic domain, which physically acts as a flexible flap. PDE3A has three isoforms.
(PDE3A1, PDE3A2, and PDE3A3), which are generated by alternative start sites in their translation\(^\text{10}\). PDE3A1, the longest isoform, has the NHR1 domain, consisting of six hydrophobic regions at the N-terminus, presumed to be transmembrane domains since PDE3A1 is often localized to the membrane of the endoplasmic reticulum. This domain has three phosphorylation sites which are phosphorylated by Protein Kinase A, B and C. PDE3A1 and PDE3A2 have the NHR2 domain, which has been shown to be important for its interaction with other proteins. PDE3A3 is cytosolic, as it lacks the NHR1 domain, the NHR2 domain, and the three phosphorylation sites\(^\text{11}\). All three isoforms have been reported to have identical kinetic activity and respond in the same way to inhibitors\(^\text{12}\).

Functionally, PDE3A has a high affinity for both cyclic adenosine monophosphate (cAMP) (K\(\text{m} = 80\) nmol/L) and cyclic guanosine monophosphate (cGMP) (K\(\text{m} = 20\) nmol/L)\(^\text{13}\). PDE3A is termed a cyclic guanosine monophosphate (cGMP) inhibited cyclic nucleotide phosphodiesterase because cGMP binds to the same pocket as cAMP, but is hydrolyzed at a much slower rate and effectively inhibits the hydrolysis of cAMP\(^\text{13}\). cAMP is a ubiquitous signaling molecule involved in major cellular proliferation, differentiation, and apoptotic pathways. The level of cyclic nucleotide monophosphate (cNMP) in the cell is controlled by a balance of its rate of production by adenylate or guanylate cyclases and rate of degradation by PDEs\(^\text{14}\).

The PDE-regulated level of intracellular cAMP directly affects proteins such as ion channels, PKA, and exchange protein directly activated by cAMP (EPAC) (Figure 3). High levels of cAMP, for example, activate PKA by binding to its inhibitory subunits, rendering the catalytic subunits active\(^\text{15}\). This activated kinase transfers a phosphate
group from ATP to serine or threonine side chains of its substrates, which include transcription factors, such as cAMP response element-binding protein (CREB) involved in proliferative, metabolic, and stress-response pathways. Overall, the target proteins regulated by the level of intracellular cAMP mediate cellular activities such as proliferation, apoptosis, differentiation and vasodilation.

Figure 3: An Overview of Phosphodiesterase Biology. Phosphodiesterases hydrolyze cellular pools of cyclic nucleotides (cNMP) to product 5’-NMP. The cNMP pool directly regulates the activity of proteins involved in cellular responses such as proliferation, apoptosis, differentiation, and vasodilation. Figure adapted from Savai et al., 2010.

PDE3A plays a critical role in regulating the strength and duration of cAMP signaling. Mechanistically, PDE3A hydrolyzes the phosphodiester bond of cyclic AMP or cyclic GMP, releasing 5’-AMP or 5’-GMP as product. In the conserved active site, a water molecule is partially ionized to a hydroxide and forms a bridge between the two metal cations. This nucleophilic hydroxyl group attacks the phosphate in the cyclic phosphate ring of cAMP and breaks the ring. A hydrogen bond network through a conserved glutamate and histidine allows for the stabilization of the oxygen that the phosphorus was once bound to. Figure 4 represents the reaction mechanism of cGMP.
hydrolyzed by PDE9. Because the histidine, glutamic acid, water molecule, and metal cations are conserved in across PDE families, the mechanism can be directly applied to PDE3.

**Figure 4: Reaction Mechanism of cGMP Hydrolysis of PDE9**. Histidine, glutamic acid, and metal cations are residues highly conserved in the catalytic domain of all phosphodiesterases. The schematic depicted the progression from enzyme+substrate (E+S), to enzyme/substrate (ES), to enzyme/product (EP), to enzyme+product (E+P). Potential hydrogen bonds were represented as dashed lines. The substrate depicted is cGMP, but the same mechanism applies for cAMP. Figure from Liu et al., 2008.

**Targeting PDE3A in Disease**

PDE3 inhibitors, such as Cilostazol, are used in clinical practice to treat heart failure resulting from a reduction in myocardial contractility. Although extensively investigated, the mechanisms by which these inhibitors improve patient health are not well understood. It is thought that because these compounds inhibit the cAMP hydrolysis function of PDE3, the concentration of cAMP increases in myocytes. An increased level of cAMP increases the phosphorylation of PKA substrates, such as calcium channels in muscle cells. Calcium influx is important for systole, and therefore improves contraction of the heart. Another PDE3 inhibitor, Zardaverine acts as a positive inotropic agent on heart muscle *in vitro*. PDE3 inhibition also increases cAMP concentration in vascular smooth muscle, which relaxes the muscle and increases vasodilation. PDE3 is expressed in platelets as well, and the same inhibitors cause an
increase in cAMP concentration, which leads to decreased platelet aggregation, therefore improving cardiac health\textsuperscript{10}.

Deregulation of proliferative cAMP-PDE3A pathways has been implicated in several cancers, as inhibited PDEs can also lead to inhibition of growth and apoptosis\textsuperscript{16,22}. Yet unraveling the altered pathways is complex because PDE3A not only has several binding partners but also has unique regulation of each isoform. PDE3A has been shown to form complexes with proteins such as 14-3-3 proteins, plectin, brefeldin A-inhibited guanine nucleotide exchange proteins, and CFTR (cystic fibrosis transmembrane conductance regulator channel), but potential binding partners of PDE3A in cancer cell lines are not well understood\textsuperscript{8,23,24}.

**A Structural, Mechanistic and Functional Understanding of the Interaction of Compound 1B and PDE3A**

In this study, we attempted to elucidate the structural, mechanistic and functional effect of Compound 1B on its hypothesized target, PDE3A. We manually docked Compound 1B to a model of PDE3 in order to predict the intermolecular interactions between Compound 1B and important residues in the binding pocket on PDE3A. Because the crystal structure of PDE3A is not available, we used the crystal structure of PDE3B for this structural analysis due to high conservation in the catalytic domain (Figure 5). PDE3B was included in the mRNA expression Pearson correlation, but its expression did not correlate with cell sensitivity to Compound 1B. However, in a Caliper phosphodiesterase enzyme activity assay, both PDE3A and PDE3B were inhibited by Compound 1B at about 97% (Luc de Waal, Unpublished Data, 2013). Because Compound 1B could equally bind to PDE3B as PDE3A, we were confident that by
analyzing Compound 1B in the active site of PDE3B, we could accurately predict its binding interactions with PDE3A. The predicted binding site and intermolecular interactions between Compound 1B and PDE3B were informed by models of interactions between PDE residues and compounds Zardavarine and Cilostazol.

In order to understand the effect of Compound 1B on the cAMP hydrolysis function of PDE3A, an in vitro enzymatic activity assay was developed. Using this assay, the ability of Compound 1B to inhibit the activity of purified PDE3A was compared to known inhibitors and structural analogs of Compound 1B. Furthermore, the PDE3A activity assay was optimized for kinetic studies. Comparison of the kinetic trends of Compound 1B to those of known competitive inhibitors, Zardavarine and Cilostazol, allowed us to gain insight on the mechanism by which Compound 1B inhibits PDE3A.

Additionally, we connected our structural and mechanistic insight to the interaction between PDE3A and Compound 1B in the context of the sensitive Hela cancer cell line. We determined if a correlation existed between the in vitro potencies and the lethality of the compounds to the Hela cell line. This comparison was used to evaluate whether Compound 1B was selectively toxic to cancer cell lines because of its ability to inhibit the cAMP hydrolysis function of PDE3A or not. By studying the interaction between Compound 1B and PDE3A, we aimed to gain a better understanding of what this interaction implies about the cytotoxic effect of Compound 1B to a subset of cancer cell lines.
Figure 5: Alignment of Human PDE3A and Human PDE3B. Green bars represent conserved amino acids. There is 41.224% conservation between both full-length proteins and 75.574% conservation between both catalytic domains (residues 670-1018 marked at the start and end by black arrows).
MATERIALS and METHODS

**Modeling of the intermolecular interactions between PDE3 and small molecules:** Crystal structure of PDE3B (1SO2) was used to manually dock compounds Compound 1B, TP5, Cilostazol, and Zadarvarine, designed in ChemBioDraw13.0\textsuperscript{25} using PyMOL Molecular Graphics System\textsuperscript{26}.

**Cell culture:** Hela and MCF7 cell lines were maintained in Dulbecco’s Modification of Eagle’s Medium (Corning Cellgro) and A549, PC3, H1563, H2122 and COLO741 cell lines in Rosewell Park Memorial Institute (Corning Cellgro). All media was supplemented with 10% FBS, and 1% penicillin and streptomycin and all cells were maintained at 37°C.

**Compounds:** Compound 1B (R), Compound 1B (S), TP5, TP6, TP7, TP26, TP38, TP43, TP49, TP50 and TP51 were synthesized by Tim Lewis. All other chemicals were purchased: Zardavarine (Sigma-Aldrich, Catalog #Z3003), Cilostazol (Sigma-Aldrich, Catalog #C0737), Levosimendan (Sigma-Aldrich, Catalog #L5545), Milrinone (Sigma-Aldrich, Catalog #M4659), Sildenafil (Sigma-Aldrich, Catalog #PZ0003), Trequinsin (Sigma-Aldrich, Catalog #T2057), Sigauzodan (Tocris Biosciences, Catalog #1148), and OR1896 (SantaCruz, Catalog #212689). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C.

**Cell viability assay in cell lines:** A 40 µL cell suspension was added to wells of a 384-well plate. After 24 hours, cells were treated with 0.1 pM to 300 mM Compound 1B, analogs, or other selected PDE3A inhibitors. 48 hours after drug treatment, 10 µL of 25% Cell Titer-Glo Luminescent Cell Viability Assay (Promega) and 75% phosphate-buffered saline at room temperature was added and ATP fluorescence was read on the EnVison 2104 Multilabel Reader (PerkinElmer).

**PDE3A enzymatic activity assay in vitro** General Protocol: The assay was developed based on PDE-Glo Phosphodiesterase Assay (Promega, Catalog #V1361). Assay reagents were dissolved in Reaction Buffer (Promega). 1 µL of Reaction Buffer and 2.5 µL of 5X Phosphodiesterase 3A (SignalChem, Catalog #P91-31G) enzyme was added to the reaction well of a 384 well plate. 2.5 µL of 5X cAMP was then added and incubated on the plate shaker for 20 minutes at room temperature. As the reaction progressed, PDE3A hydrolyzed cAMP. 2.5 µL of Termination Buffer (containing a high concentration of IBMX) was added to the reaction well to completely inhibit the active PDE3A after 30 minutes immediately followed by 2.5 µL of Detection Solution containing inactive PKA and ATP. The remaining cAMP that was not hydrolyzed by the PDE3A activated PKA by binding to its regulatory subunits and releasing its catalytic subunits. Active PKA then transferred a phosphate group from ATP to substrate, depleting the concentration of ATP. 10 µL of Kinase-Glo (Promega) was added to the reaction well and incubated on the plate shaker for 10 minutes at room temperature in the dark. The Kinase-Glo reagent contains luciferase, which uses the remaining ATP and
converts luciferin to oxyluciferin and in the process generates light proportional to the amount of ATP remaining. This reaction is a measurement of the progression of the PKA catalyzed reaction because it measures remaining reactant ATP. The luminescence was measured on the Spectramax luminometer with an integration time of 1 second/well. Luminescence values were normalized to the appropriate control wells. Controls for each experiment: 1) Reaction buffer as a blank; 2) No PDE3A or inhibitor as a low luminescence signal; 3) No cAMP as a high luminescence signal; 4) DMSO.

A range of concentrations of PDE3A and cAMP as well as incubation times were tested separately with otherwise identical reaction conditions to determine the experimental conditions resulting in the greatest dynamic range of signal. Schematic representation of the assay and representative luminescence output depicted in Figure 6 below.
Figure 6: Schematic of PDE3A Enzymatic Activity Assay. A) The reaction and detection of the activity of PDE3A. B) Representative luminescence signal from control conditions for assay. As cAMP concentration increased in the reaction well, the luminescence decreased linearly.
**Inhibitor assays:** Assays were performed using the General Protocol with 1 µL of 5X Compound 1B (racemic mixture and S- & R-enantiomer), known PDE inhibitors (Zardavarine, Cilostazol, Trequinsin), and analogs of Compound 1B (TP5, TP6, TP7, TP26, TP38, TP43, TP49, TP50 and TP51) dissolved in DMSO. The inhibitors were added to reaction wells containing Reaction Buffer and PDE3A at a final concentration of 0.1% for a 10-minute incubation on a plate shaker, before proceeding to the rest of the assay described in the General Protocol. The accumulated pool of cAMP, due to the inhibition of PDE3A hydrolysis of cAMP to 5’-AMP in the well, lowers the luminescence signal that is recorded on the Spectramax Luminometer. Luminescence values for each concentration were divided by the DMSO control luminescence average, and were plotted as percentages at each concentration of inhibitor using GraphPad PRISM software. The half-maximal concentration to inhibit the cAMP hydrolysis activity of PDE3A, or the IC$_{50}$ values, were calculated with GraphPad PRISM software to compare the potencies of inhibitors.

**Kinetics Experiments:** Assays were performed using the General Protocol with slight modifications. First, Reaction Buffer, PDE3A (285 pg/µL), and the inhibitor were incubated for ten minutes, and then cAMP (0.3 µM) was added to start the reaction. After precisely two minutes, termination buffer was added to arrest the reaction. The General Protocol was followed for the completion of the assay. Inhibitor concentrations were chosen in a narrow range close to the IC$_{50}$ value as determined by the in vitro Inhibitor Assay. Luminescence was plotted against cAMP concentration for each concentration of inhibitor using GraphPad PRISM software.

Luminescence output data was converted to velocity, for Michaelis-Menten visualization. This conversion was based on the following calculations. Luminescence units were converted to velocity of the reaction by first calculating the amount of 5’-AMP product generated at each concentration of cAMP added and next dividing the product by the time of the reaction for velocity. Example calculations for Zardavarine were used for the following transformation of assay output to a Michaelis-Menten visualization. Cilostazol and Compound 1B were subsequently analyzed in the same way.
Figure 7: The Normalized cAMP Standard Curve. A. Luminescence values at each concentration of cAMP were converted from arbitrary units to a scale from 0 to 1. B. The data was transformed to log[cAMP] and a sigmoidal fit was assigned using PRISM software.

The standard curve of the luminescence value at each concentration of cAMP was normalized so luminescence was measured as a function from 0 to 1, where \( L_x \) represented each data point collected, \( L_0 \) represented the lowest luminescence signal at \([cAMP] = 1 \mu M\), and \( L_{\text{max}} \) represented the highest luminescence signal where \([cAMP] = 0 \mu M\). This cAMP standard curve was created following the General Protocol, with the exception of adding Reaction Buffer instead of 1.5 \( \mu L \) of PDE3A in Reaction Buffer. Each point represented the luminescence signal that corresponded to each cAMP. The \([cAMP]\) values were transformed to log([cAMP]). This transformation was applied in order to expand the visualization of the data points at very low concentrations of cAMP. The sigmoidal curve (Sigmoidal, \( X \) is log concentration) was applied using PRISM software based on the trend of the data (Figure 7).

\[
\frac{L_x - L_{\text{min}}}{L_{\text{max}} - L_{\text{min}}} = \text{Normalized Luminescence}
\]

\[
\begin{align*}
\text{at } [cAMP] &= 0 \mu M & \frac{146,330 - 52,280}{146,330 - 52,280} &= 1 \\
\text{at } [cAMP] &= 1 \mu M & \frac{52,280 - 52,280}{146,330 - 52,280} &= 0
\end{align*}
\]

Next, the average of the four replicate values of luminescence at each concentration of Zardavarine was calculated. Averages were chosen instead of individual replicates to facilitate the interpolation. These average values were normalized in the same manner where the maximum luminescence in the assay was 300,153 units at \([cAMP] = 0 \mu M\), \([\text{Zardavarine}] = 0 \mu M\) and the minimum luminescence was 440 units at \([cAMP] = 1 \mu M\), \([\text{Zardavarine}] = 0 \mu M\).
\[
\begin{aligned}
\text{at} \ [\text{cAMP}] = 0 \mu\text{M} & \quad \frac{300,153 - 440}{300,153 - 440} = 1 \\
\text{at} \ [\text{cAMP}] = 1 \mu\text{M} & \quad \frac{440 - 440}{300,153 - 440} = 0
\end{aligned}
\]

These luminescence values for Zardavarine were translated to their corresponding [cAMP]. The values of normalized luminescence were inputted to the normalized cAMP standard curve and values of corresponding [cAMP] were taken as output. These output values represent the [cAMP]c once the reaction was terminated after two minutes. Extremely high values of normalized luminescence approaching a value of 1 could not be interpolated because the concentration of cAMP approached 0 and these data points were therefore excluded. The [cAMP]i was the concentration added to the reaction well at the start of the reaction. Therefore, the difference between these two values gave the concentration of the 5'-AMP product formed.

\[
\text{[product]} = [\text{cAMP}]_i - [\text{cAMP}]_f
\]

The [product] was divided by a 2 minute reaction time, yielding velocity (5'-AMP \mu\text{M/min}).

\[
\text{velocity} = \frac{\text{[product]}}{\text{time}}
\]

The 2 minute reaction time was chosen because at all concentrations of cAMP, there was still cAMP remaining (Figure 8A), in other words, not all 5'-AMP was formed from the inputted cAMP (Figure 8B). This indicated that there was plenty of available substrate for the PDE3A hydrolysis reaction. At the 4 minute timepoint, there was only remaining cAMP substrate at 2 \mu\text{M} cAMP, and at the 30 minute timepoint, all [cAMP] was entirely consumed by the reaction. Because reactions at 2 minutes still had substrate present, this timepoint was chosen for the subsequent kinetics studies. Any shorter reaction time was not manually feasible within the limitations of the assay.

Kinetic trends were observed using this visualization comparing known competitive inhibitors Zardavarine and Cilostazol to Compound 1B. Data was visualized as Michaelis-Menten plots where reaction velocity was plotted against initial substrate concentration. A two-step reaction was assumed: cAMP binds to PDE3A and then the cAMP substrate is hydrolyzed to 5’-AMP and released as product. One assumption of Michaelis-Menten was that after reactions were terminated, there were still saturating substrate concentrations. As seen in Figure 8, at each substrate concentration, there was remaining cAMP substrate. Every PDE3A molecule therefore had substrate bound. This PDE3A/cAMP complex was in steady state where the concentration was constant throughout the reaction. We made assumptions that the binding step of cAMP to PDE3A was fast and the system quickly reached equilibrium, while the catalytic step was rate-limiting.
Figure 8: Remaining [cAMP] After 2, 4, and 30 Minute Reaction Times. A range of concentrations of cAMP from 0.0 µM to 2.0 µM initiated the reaction with 0.2 µM of PDE3A. Reactions were terminated at 2, 4, and 30 minute time points. A. The remaining [cAMP] at each initial [cAMP] was plotted. B. Alternative visualization where [5’-AMP] was plotted at each initial [cAMP]. The dotted line at y=x represented the linear relationship between the complete hydrolysis of cAMP to 5’-AMP.
RESULTS

In this study we carried out a comparative analysis of the interaction between PDE3A & Compound 1B and known PDE inhibitors (Cilostazol and Zardavarine) in order to gain a structural and mechanistic understanding of how Compound 1B interacts with PDE3A. This analysis was applied to cancer cell lines in order to make conclusions about the functional effect of the interaction between Compound 1B and PDE3A on the cAMP hydrolysis activity of PDE3A and about the selective toxicity of Compound 1B in cancer cell lines.

Visualization of Compound 1B Bound to the Catalytic Site of PDE3B

In the absence of a crystal structure for PDE3A, the use of the existing PDE3B structure (1SO2) for this study is justified due to the high sequence homology between PDE3A and PDE3B in the catalytic domain (Figure 5). In order to visualize the molecular interactions of Compound 1B and PDE3B, a two-pronged approach was taken: first, the structural commonalities between cAMP, Zardavarine, Cilostazol, Compound 1B and analog TP5 were analyzed; and second, conserved intermolecular interactions between models and co-crystal structures of cAMP/PDE3A\(^27\), Cilostazol/PDE3A\(^27\), and Zardavarine/PDE4D (PDB: 1XOR) were used as a guide to manually dock Compound 1B in the crystal structure of PDE3B. Structural and chemical similarities between cAMP, Zardavarine, Cilostazol, Compound 1B, and TP5 were analyzed in order to align the compounds and better understand the potential for interactions in the catalytic site of PDE (Figure 9). Zardavarine, Cilostazol, Compound 1B, and TP5 share pyridazinone and benzene rings. The partially negative charge of the carbonyl oxygen on the
pyridazinone ring is chemically similar to the negatively charged phosphate group of cAMP. The presence of hydrogen bond donors and acceptors allows for specificity and strong interactions in the binding site; the oxygen and nitrogen of the pyridazinone ring acts as potential hydrogen bond acceptors, and the nitrogen of the (NH) acts as a potential hydrogen bond donor. The adenine base of cAMP has the potential to take part in several hydrogen bonds, similar to the variable substituents of the benzene ring of the inhibitors. The benzene ring of the inhibitors acts as a spacer between the pyridazinone ring and the substituents of the benzene ring to mimic the furan ring of the ribose sugar in cAMP that bridges the phosphate to adenine. The spacer could orient the substituents and the pyridazinone ring to facilitate their specific interactions. These parallels between the inhibitors and cAMP provide chemical reasoning that all small molecules are equipped to take part in similar interactions in the catalytic domain of PDE3.
Figure 9: Chemical and Structural Similarities of Compound 1B, Analog TP5, PDE Inhibitors Zardavarine and Cilostazol, and Natural Substrate cAMP. Portions of the molecules were colored for comparison. The adenine of the cAMP molecule and the substituents of the benzene rings of Compound 1B, TP5, Zardavarine and Cilostazol were colored red. The furan ring of cAMP and the benzene rings of the other compounds were colored green. The negatively charged phosphate group and the partially negative carboxylic oxygen were colored blue. The pyridazinone rings of Compound 1B, TP5 and Zardavarine were highlighted in pink, as was the similarly structured ring of Cilostazol.

Previously modeled compound/PDE complexes were analyzed in order to guide manual docking of Zardavarine, Cilostazol, Compound 1B, and TP5 in the structure of PDE3B. Figure 10 highlights the key interactions between a model of cAMP and Cilostazol bound to PDE3A and a co-crystal structure of Zardavarine bound to PDE4D. Substrate binding includes an electrostatic interaction between the phosphate group of cAMP and the coordinated Zn$^{2+}$, and a hydrogen bond with His752 (Figure 10A). The furan ring of the ribose interacts with Tyr751, Leu910, and Ile968 through van der Waals forces. The adenine base is hydrophobic, and is positioned near hydrophobic amino acids Phe972, Phe989, and Phe1004.
Figure 10: Model of cAMP and Cilostazol in Catalytic Site of PDE3A$^{28}$ and Zardavarine in Catalytic Site of PDE4D. Previous models and co-crystal structures were used to identify conserved interactions. A. Key interactions between cAMP and Cilostazol compounds and PDE3A were conserved in our structural study. The blue ribbon highlighted the secondary structure of the binding pocket while the orange sticks highlighted amino acids in the binding site. The Zn$^{2+}$ was represented as a blue hexagon. cAMP was marked in green and Cilostazol was marked in purple. B. Zardavarine crystallized in the catalytic site of PDE4D (PDB: 1XOR). PDE3B was visualized using cartoon representation of the amino acid backbone. The oxygen atoms were colored red and the nitrogen atoms were colored blue. The carbon backbone was colored pink. Conserved residues important for binding, Ile 336, Gln 369 and Phe 372, and the magnesium and zinc cations were highlighted in blue.
When analyzing the interactions between inhibitors (Zardavarine and Cilostazol) and the PDE catalytic domain, it became apparent that features of the catalytic site important for binding cAMP were also critical for interactions with the inhibitors due to their structural similarities. The compounds were inserted into PDE3B by maintaining key interactions in the models between compounds and residues conserved across PDEs. Because Zardavarine and Cilostazol are bound in the active site of the PDEs when modeled, Compound 1B and analog TP5 were built into the same binding site of PDE3B (Figure 11). These conserved interactions are described in the context of Compound 1B, but were based on the conserved interactions between residues of the catalytic domain of PDE and compounds Zardavarine, Cilostazol, and cAMP.

A conserved isoleucine and phenylalanine create a hydrophobic clamp, which sandwiches and favorably interacts with the planar benzene ring of Compound 1B (Figure 11A). This hydrophobic interaction is not specific, but is entropically favorable for the system overall, as it minimizes direct contact with water. The amine functional group of glutamine has the potential to make specific hydrogen bonds with the substituents off of the benzene ring, specifically the nitrogen of the diethylamine of Compound 1B. The polar nitro substituent on Compound 1B remains solvent exposed, oriented in the opposite direction of the hydrophobic protein core. The negatively charged oxygen of the pyridazinone ring is electrostatically attracted to the Mg^{2+} bound in the active site. Because Compound 1B, TP5, Zardavarine and Cilostazol share these chemical and structural features important for maintaining interactions with the conserved residues in the PDE active site, we hypothesized that the compounds bound in a similar fashion (Figure 11C).
As seen with Zardavarine and Cilostazol, when Compound 1B and TP5 are bound to the active site of PDE3, cAMP cannot bind to the active site at the same time. This comparative visualization of these complexes along with chemical reasoning implied a competitive binding mechanism of Compound 1B.
Figure 11: Models of Compound 1B, Analog TP5 and Inhibitors Zardavarine and Cilostazol Interacting with Conserved Residues in the Catalytic Domain of PDE3. The crystal structure for PDE3B (1SO2) was used to create these models. PDE3B was visualized using an overlay of the surface representation with cartoon representation of the amino acid backbone beneath. The oxygen atoms were colored red and the nitrogen atoms were colored blue. The carbon backbone was colored pink. Conserved residues important for binding, Ile 955, Gln 988 and Phe 991, and the magnesium cations were highlighted in blue. A. Compound 1B modeled in the binding pocket of PDE3. B. Zoomed in portion of the catalytic domain with Compound 1B. C. Comparison between the binding of Compound 1B to Zardavarine, Cilostazol and TP5.

Optimization of PDE3A Activity Assay for *in vitro* Analysis of the Interaction Between PDE3A and Compound 1B

In order to investigate the interaction between Compound 1B and PDE3A empirically, a PDE3A activity assay was developed to measure the cAMP hydrolysis activity of PDE3A alone and in the presence of a panel of compounds. The catalytic domain, of PDE3A (amino acids 641-1009) was used for the developed assay that was based on PDE-Glo Phosphodiesterase Assay by Promega. While buffers, reagents, and protocols were provided with the assay, the concentrations of PDE3A and cAMP used in the assay, and the duration of the reaction needed to be optimized (Figure 12).

Figure 12: Optimization of the PDE3A Activity Assay. A. Optimization of reaction time and concentration of PDE3A. B. Optimization of concentration of cAMP. C. Dynamic range of the optimized assay. At high (1 µM) and low (1 nM) concentrations of Compound 1B ((S) and (R) enantiomers), Trequinsin, and Sildenafil, the luminescence was recorded. Controls of Reaction Buffer, PDE3A & DMSO, cAMP & DMSO, and cAMP, PDE3A & DMSO were included. Reaction time of 30 minutes, and concentrations of 285 pg/µL PDE3A and 0.3 µM cAMP were selected to ensure that experimentation was conducted in the dynamic range of the assay.
A range of concentrations of PDE3A and cAMP as well as incubation times were tested separately with otherwise identical reaction conditions to determine the experimental conditions resulting in the greatest dynamic range of signal. The EC$_{50}$ of PDE3A determined to be 285 pg/µL was set as the optimum PDE3A concentration for further experiments. This value was in the steep linear section of the curve ensuring high sensitivity in the assay, since slight inhibition of the enzyme would show a large decrease in luminescence (Figure 12A). The recorded luminescence reached a maximum at 30 minutes and stayed consistent for up to 2 hours at room temperature (Figure 12A); therefore a reaction time of 30 minutes was chosen for subsequent experiments. The optimal cAMP concentration was determined to be 0.3 µM based on luminescence readings after serial dilutions of cAMP was incubated with 285 pg/µL of PDE3A (Figure 12B). This concentration was in the dynamic range of the assay and any higher concentration reached a plateau of zero luminescence ensuring that any decrease in the concentration of cAMP by PDE3A hydrolysis would result in a drastic increase in luminescence from that of the 0.3 µM cAMP concentration.

Once the reaction time, PDE concentration, and cAMP concentration were optimized, the dynamic range of the luminescence signal produced by the assay was tested for Control conditions as well as several inhibitors at both high (1 µM) and low (1 pM) concentrations (Figure 12C). When the DMSO & cAMP only control condition was compared to the DMSO, cAMP & PDE3A experimental condition, a drastic increase in luminescence signal was observed, indicating the hydrolysis of cAMP by PDE3A. Low doses of compounds did not alter the luminescence of the reaction, indicating a fully active PDE3A, comparable to the control condition without inhibitor. High doses of
Compound 1B (R), Trequinsin and Sildenafil all reduced the activity of the protein. The (R) and the (S) enantiomers of Compound 1B, rather than the racemic mixture, were tested separately in this assay in order to determine if this stereochemistry was important in PDE3A binding. When PDE3A was incubated with Compound 1B (S) at a high dose, its activity remained high indicating that Compound 1B (S) was incapable of inhibiting PDE3A. These results suggested that Compound 1B (R), like known PDE3A inhibitors, inhibited the cAMP hydrolysis function of PDE3A.

In addition to determining whether compounds inhibited PDE3A or not, we were curious as to the relative potencies of Compound 1B, analogs, and known PDE3A inhibitors. We therefore performed an activity assay with a range of inhibitor concentrations. As the concentration of Compound 1B (R) was increased from 0.2 µM to 3 µM, the cAMP hydrolysis activity of PDE3A was reduced from close to 100% to close to 0% (Figure 13). The IC$_{50}$ value was determined to be 1.480 µM. As expected, Compound 1B (S) did not reduce the activity of PDE3A across these extended concentrations. This result that PDE3A activity was inhibited by Compound 1B (R) in a dose-dependent manner confirmed previous results of inhibition at a high, but not a low dose.
Figure 13: The Effect of Compound 1B (R) and (S) on the cAMP Hydrolysis Function of PDE3A. 0.2 µM to 3 µM of Compound 1B (R) and (S) were incubated with PDE3A for 10 minutes before initiating the hydrolysis reaction with the addition of cAMP, following the General Protocol. The % Activity of PDE3A was determined by dividing the luminescence output at each concentration by the highest luminescence signal at 0 µM of compound. Error bars represented the standard deviation of four replicates.

In order to determine how the potency of Compound 1B and structural analogs compare to PDE3 inhibitors, IC$_{50}$ values were reported for analogs of Compound 1B and known PDE3 inhibitors (Figure 14). Some structural analogs of Compound 1B, such as TP51, inhibited the cAMP hydrolysis activity of PDE3A at a higher potency than Compound 1B, with an IC$_{50}$ value of 0.256 µM. Other analogs, such as TP7 did not inhibit the cAMP hydrolysis activity of PDE3A at any concentration tested. Trequinsin, a known PDE3 inhibitor, inhibited the activity of PDE3A at lower concentrations than Compound 1B, with an estimated IC$_{50}$ value of 0.007 µM. Other known PDE3 inhibitors, Zardavarine and Cilostazol had IC$_{50}$ values of 20 µM and 130 µM respectively. IC$_{50}$ values determined from this assay were compared to IC$_{50}$ values in the literature (Figure 14D). The literature reported IC$_{50}$ values for Zardavarine and Cilostazol are
approximately a thousand fold smaller than found using our enzymatic activity assay, whereas Trequinsin was a hundred times smaller than we report. This discrepancy is likely due to different assay conditions, especially variable PDE3A concentration. As seen in Figure 14D, Compound 1B was a potent inhibitor of the cAMP hydrolysis activity of PDE3A with a 1.48 µM IC$_{50}$, ranking fifth among the fourteen tested analogs and known inhibitors.

![Figure 14: The Effect of Compound 1B, Analogs, and Known Inhibitors on the cAMP Hydrolysis Function of PDE3A.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>Reported IC$_{50}$ value (µM)</th>
</tr>
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<td>Compound 1B (R)</td>
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</tr>
<tr>
<td>Compound 1B (S)</td>
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</tr>
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</table>

*Lethal*
Compound 1B including TP5, TP6, TP7, TP26, TP38, TP43, TP49, TP50 and TP51.  B. Cilostazol.  C. Zardavarine.  The % Activity of PDE3A was determined by dividing the luminescence output at each concentration by the highest luminescence signal at 0 µM of compound. A “log (inhibitor) v. response – Variable slope (four parameter)” curve was fit to the data using PRISM software and this curve was used to determine the IC\textsubscript{50} values summarized in D. Reported IC\textsubscript{50} values from the literature were recorded for applicable compounds.

**Compound 1B Followed Kinetic Trends of Competitive Inhibitors of PDE3A**

In order to determine the effect of Compound 1B on the kinetics of the cAMP hydrolysis function of PDE3A, a comparative study of the kinetics with two known competitive inhibitors of PDE3A, Zardavarine and Cilostazol, was performed. Both our modeling and binding studies suggested that Compound 1B binds at a very similar site to cAMP and competitive inhibitors of PDE3A. Therefore, we tested whether kinetic trends of the hydrolysis reaction incubated with Compound 1B were also similar to those of the competitive inhibitors Zardavarine and Cilostazol. Luminescence output of the kinetics assay was transformed to a Michaelis-Menten visualization to plot our kinetics data where the velocity of the reaction was plotted against substrate concentration. Adhering to the assumptions of the Michaelis-Menten model, we expected to determine kinetic parameters $V_{\text{max}}$ and $K_m$, where $V_{\text{max}}$ is the maximum velocity of the PDE3A hydrolysis reaction with infinite cAMP, and $K_m$ is value the concentration of cAMP substrate required for hydrolysis to occur at half the maximal rate. Therefore a high $K_m$ value implies a higher concentration of substrate is necessary to achieve the half maximal rate and a weak binding of substrate to protein. The presence of a competitive inhibitor effectively lowers the affinity of PDE3A for the cAMP; increasing concentrations of inhibitor increase the $K_m$. On the other hand, because infinite substrate will overcome the
effect of the competitive inhibitor, the maximum velocity that the enzyme can hydrolyze cAMP will not change with inhibitor present.

At the time of experimentation, the unreacted input cAMP confounded the change in signal of reacted cAMP hydrolyzed into 5’-AMP product. When the kinetics data was visualized as velocity versus initial substrate concentration, it was apparent that the $V_{\text{max}}$ plateau was not reached (Figure 15A). Although the kinetic parameters of $V_{\text{max}}$ and $K_m$ could not be determined, trends of the two competitive inhibitors were still apparent (Figure 15). These quantitative trends are summarized in Table 1. Because inhibitor concentrations for the kinetics experiments were chosen in close proximity of the IC$_{50}$ values, ratios of [Inhibitor] to their IC$_{50}$ values were calculated for facilitated comparison. At low concentrations of cAMP, as inhibitor concentration increased, the velocity of the reaction decreased (Figure 15B and C). At initial [cAMP] = 0.5 µM, as the concentration of Zardavarine was increased from 0.337 of its IC$_{50}$ value to 0.867 of its IC$_{50}$ value, the velocity decreased from 0.076 µM 5’-AMP formed/min to 0.051 µM/min (Table 1). Similarly, as the concentration of Cilostazol rose from 0.307 of its IC$_{50}$ value to 0.922 of its IC$_{50}$ value, the velocity decreased from 0.055 µM 5’-AMP formed/min to 0.053 µM/min (Table 1). In the uninhibited reactions, where concentrations of Zardavarine and Cilostazol were 0 µM, the velocity of the reaction was higher than any inhibitor concentration at 0.239 µM/min and 0.238 µM/min respectively (Table 1). At low substrate concentrations, the reaction rate was decreased because the inhibitor effectively replaced the substrate at the binding site and hindered hydrolysis. At higher initial concentrations of cAMP, such as 2 µM, the substrate was in excess compared to the inhibitor, and the reaction was essentially uninhibited. At any concentration of
Zardavarine and Cilostazol, a velocity at 0.8 µM/min was reached (Table 1). Even though all inhibitor concentrations converged at this velocity at 2 µM cAMP, a $V_{\text{max}}$ could not be determined without the presence of a plateau at this point (Figure 15A, B and C). These previously reported competitive inhibitors, Zardavarine and Cilostazol, exhibited the trends of competitive inhibitors in this PDE3A enzymatic activity assay.

The same analysis was repeated for Compound 1B, and we found that Compound 1B followed the kinetic trends observed for competitive inhibitors, Zardavarine and Cilostazol (Figure 15D and Table 1). At 0.5 µM cAMP, as the concentration of Compound 1B increased, the velocity of the reaction decreased. As the concentration of Compound 1B rose from 0.203 of its IC$_{50}$ value to 0.676 of its IC$_{50}$ value, the velocity decreased from 0.103 µM 5'-AMP formed/min to 0.052 µM/min (Table 1). Additionally, at 2 µM cAMP, any concentration of Compound 1B reached a velocity at 0.8 µM/min (Table 1). Because the kinetic trends of a competitive inhibitor match those of Compound 1B, we concluded that Compound 1B behaved similarly when interacting with PDE3A.
Figure 15: Visualization of Kinetics Data as Michaelis-Menten Plots for Competitive Inhibitors Zardavarine and Cilostazol, as well as Compound 1B. A range of concentrations of Zardavarine, Cilostazol, and Compound 1B were incubated with PDE3A for 10 minutes before initiating the hydrolysis reaction with the addition of cAMP, following the General Protocol. Reactions were incubated for 2 minutes before terminated with Termination Buffer. Luminescence was measured as output for each reaction as seen for Zardavarine in A. On the x-axis, [cAMP] represented initial substrate added to the reaction. The luminescence values along the y-axis were a measure of total [cAMP] remaining after the termination of the reaction, obscuring the change in [cAMP] over the course of the reaction. Error bars represent standard deviation of four replicates. B, C and D. In order to visualize Michaelis-Menten plots as velocity of 5’-AMP produced during the reaction versus initial substrate concentration, luminescence output.
was transformed into a readout of product by subtracting the luminescence of cAMP without enzyme present from the luminescence of cAMP remaining after the reaction incubated with Zardavarine, Cilostazol and Compound 1B. This difference in luminescence signal was converted to velocity. The x-axis represented initial [cAMP] added to the reaction. For the transformation, averages of luminescence were taken instead of all replicates, hence there are no error bars. Several values of luminescence could not be converted to concentrations of 5’-AMP because these luminescence were so close to 1, thus concentrations of cAMP approached 0.

Table 1: Quantification of the Reaction Velocity at Various Inhibitor and cAMP Concentrations.

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<th>Compound</th>
<th>IC$_{50}$ µM</th>
<th>[Inhibitor] µM</th>
<th>[Inhibitor]/ IC$_{50}$</th>
<th>Velocity at 0.5 µM cAMP (µM/min)</th>
<th>Velocity at 2.0 µM cAMP (µM/min)</th>
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Rescue by Non-Lethal Compounds Indicated Competitive Inhibition in the Sensitive Hela Cell Line

After determining that PDE3A was the single gene whose expression correlated with cellular sensitivity to Compound 1B, Compound 1B structurally and chemically “fit” in the binding site of PDE3A, and Compound 1B inhibited PDE3A in vitro following kinetic trends of known competitive inhibitors, we hypothesized that Compound 1B competitively inhibited PDE3A in cells. In order to see evidence of
competitive inhibition in the sensitive Hela cell line, inhibitors that were not lethal to the Hela cell line, but that did inhibit PDE3A \textit{in vitro} were co-treated with TP30, a potent analog of Compound 1B (Luc de Waal, Unpublished Data, 2013). These non-lethal competitive inhibitors, Trequinsin, Milrinone, Cilostazol, Levosimendan, OR-1896 and Siguazodan, rescued the cell-death phenotype when co-treated with TP30 as seen by the right shift in the EC$_{50}$ of the treatment with TP30 alone (Figure 16). The non-lethal inhibitors essentially diluted the effect of the potent and lethal analog of Compound 1B. TP30 and by extension, Compound 1B, were competing for the same active site as the non-lethal competitive inhibitors. This experiment connected our observation that Compound 1B was a competitive inhibitor of PDE3A \textit{in vitro} with the observation that this competitive relationship held in the Hela cell line and was related to the cell lethality phenotype.
Figure 16: Non-lethal PDE Inhibitors to Hela Cells Rescue the Lethal Effect of Compound 1B. A. Cells were co-treated with Compound 1B and 10 µM of non-lethal Cilostazol, Levosimendan, OR1896, Siguazodan, or Milrinone. After 48 hours of treatment, cell viability was assessed using the Cell Titer-Glo assay. B. Structures of the non-lethal inhibitors (Luc de Waal, Unpublished Data, 2013).

Comparison of the in vitro Potencies to the in vivo Lethality of Compound 1B, its Structural Analogs and Known PDE Inhibitors

In order to determine if the ability of Compound 1B to inhibit the cAMP hydrolysis activity of PDE3A was sufficient to explain its lethality in cancer cells, the in vitro potencies were compared to the ability of the compounds to induce selective cell death. Compounds were divided into categories of combinations of in vitro (binder or non-binder) and cell lethality behavior (lethal or non-lethal): 1) lethal binders to PDE3A, 2) non-lethal binders, and 3) non-lethal non-binders (Figure 17). Lethal compounds TP26 and TP43 did not inhibit PDE3A in vitro at the concentrations tested. We hypothesize that because these structures do not have all functional groups present in Compound 1B, they cannot make as strong interactions with PDE3A. Just as high
relative concentrations of TP26 and TP43 were needed to cause Hela cell death, we assume that a higher concentration of these two compounds *in vitro* would inhibit PDE3A. TP7, Compound 1B (S), and TP50 were non-lethal non-binders of PDE3A, indicating the limited flexibility of compounds to bind to the catalytic domain. Compounds lethal to the Hela cell line bound and inhibited PDE3A *in vitro*. However, Trequinsin, a non-lethal binder, inhibited the cAMP hydrolysis function of PDE3A more potently than Compound 1B, a lethal binder. This indicated that an optimal PDE3A inhibitor did not imply an optimal cytotoxic agent. Compound 1B was modeled as bound to the catalytic site of PDE3A through comparison to known PDE3 inhibitors, structural analogs, and non-lethal PDE3 inhibitors. In addition, Compound 1B was empirically validated as competing for this binding site *in vitro* and in the Hela cell line. Because non-lethal PDE3 inhibitors rescued the lethal effect of Compound 1B, competitive binding was directly linked to the cytotoxic effect of Compound 1B.
Figure 17: Three Categories of Compounds Derived from Inhibitor Potencies \textit{in vitro} (binders or non-binders) and Their Ability to Induce Selective Toxicity in Cancer Cell Lines (lethal or non-lethal). Reported effective concentrations of compounds required to reduce cell viability by 50% (EC$_{50}$).
DISCUSSION

In this study we validated and characterized the effect of Compound 1B, a novel cytotoxic agent, on the enzymatic activity of its proposed molecular target, phosphodiesterase 3A (PDE3A). Compound 1B shared chemical and structural similarities with other PDE inhibitors Zardavarine and Cilostazol. The structural conservation of the PDE3A inhibitors was used to model Compound 1B and analog TP5 into the binding site of the highly conserved PDE3B, in order to visualize the Compound 1B/PDE3A complex and predict important interactions for binding. These similarities proved to indicate mechanistic similarities as well. When looking at the effect of Compound 1B on the kinetics of the cAMP hydrolysis of PDE3A, it was determined that Compound 1B, like Zardavarine and Cilostazol, competed with cAMP for the catalytic domain of PDE3A.

An understanding of the empirical data and theoretical models of the effect of these compounds on PDE3A in vitro was juxtaposed with the lethality data in cell lines to infer the functional implications of this binding. Known PDE3A inhibitors Trequinsin, Cilostazol were not lethal to cells, but were still extremely potent inhibitors of the enzymatic activity of PDE3A. The existence of this non-lethal binder category of compounds implies that simply binding to PDE3A at the catalytic site is not sufficient to induce the lethality of Compound 1B in cells. Additionally, because Compound 1B competitively inhibits cAMP hydrolysis activity of PDE3A as does lethal Zardavarine and non-lethal Cilostazol, competitively inhibiting the hydrolysis is also not sufficient for the selective toxicity of Compound 1B in cell lines. The prediction that the binding of
Compound 1B to PDE3A in vitro was confirmed in the Hela cell line because non-lethal PDE3A competitive inhibitors rescued the lethal effect of Compound 1B. Although Compound 1B induces its effect by binding to the catalytic site of PDE3A, its selective toxicity is likely due to some other mechanism derived from this complex aside from inhibiting the cAMP hydrolysis function of PDE3A.

The structural investigation of Compound 1B bound to the catalytic domain of PDE3A prompted speculation as to how changes to Compound 1B would affect binding. Because space and favorable interactions in the active site are limited, structural analogs of Compound 1B, which deviate significantly from the conserved backbone, are not predicted to bind to PDE3. The pyridazinone is conserved among compounds, and modifications (such as those present in TP7, TP50, and Compound 1B (S)) are therefore expected to prevent their binding (Figure 18, circled in white). The amide nitrogen provides strong electron donation to the carbonyl oxygen; replacing the nitrogen with a carbon would no longer provide electron density to the carbonyl oxygen. Atomic changes to the pyridazinone ring, such as replacing the NH proton with a methyl group, are expected to disrupt chemical integrity and interactions necessary for binding. Additionally, modifying atoms that have the ability to participate in hydrogen bonds would eliminate this binding potential. For example, replacing the amide hydrogen with a methyl group would eliminate a specific hydrogen bond. Replacing the nitrogen with a carbon would eliminate the hydrogen bond possibility as well. The atoms of the pyridazinone are presumably important for binding, as changes are expected to decrease binding. Replacing any nitrogen with oxygen would eliminate a hydrogen bond donor and add an acceptor. Although variability in the substituents on the benzene ring was
tolerated for binding, larger modifications would presumably lead to steric hindrance in the binding site of PDE3 (Figure 18, circled in yellow). Large substituents would not be able to exist in the same space as residues at the edge of the binding pocket, such as glutamine. The chemical space that the compounds will be able to bind is limited by the chemical nature and size of the active site. The importance of these chemical and structural modifications could be validated by mutating key residues in the binding site to see if elimination of an interaction also eliminates binding. Additionally, these structural analogs could be synthesized if possible and tested to see which modifications eliminate binding to PDE3A.

**Figure 18: Proposed Key Features of PDE3 Inhibitors Important for Binding to the Catalytic Site.** The crystal structure for PDE3B (1SO2) was used to create this model. PDE3B was visualized using an overlay of the surface representation with cartoon representation of the amino acid backbone beneath. The oxygen atoms were colored red and the nitrogen atoms were colored blue. The carbon backbone was colored pink. Conserved residues important for binding, Ile 955, Gln 988 and Phe 991, and the magnesium cations were highlighted in blue. The pyridazinone ring was circled in white and the substituents off the benzene ring were circled in yellow circle.
The empirical study of Compound 1B and other small molecules interacting with PDE3A *in vitro* revealed that Compound 1B follows similar kinetic trends as competitive inhibitors Zardavarine and Cilostazol (Figure 15). Although the assay used in this study was well suited for observing the effect of inhibitors on the cAMP hydrolysis activity of PDE3A, there were several limitations of the assay, particularly that arose in the kinetic study. First, the unreacted input cAMP obscured the change in signal of reacted cAMP hydrolyzed into 5’-AMP product because the assay could not distinguish the change in luminescence from before to after the reaction at each initial cAMP concentration. Only later when the product was calculated and the data was visualized as velocity versus initial substrate concentration, was it apparent that $V_{\text{max}}$ plateau was not reached (Figure 15). The presence of this plateau is necessary to establish a maximum velocity and determine other kinetic parameters such as $K_m$. In future experiments, higher initial concentrations of cAMP would be inputted until a plateau was reached to allow for the comparison of how Compound 1B and other small molecules affect the kinetics of the cAMP hydrolysis reaction of PDE3A.

Secondly, luminescence output relied on several reactions that translated the cAMP concentration left after the initial hydrolysis reaction. These three subsequent reactions were assumed to proceed as follows: 1) the addition of a large amount of IBMX was assumed to terminate the hydrolysis reaction by obliterating the activity of PDE3A; 2) all remaining cAMP was expected to activate PKA and the proportion of activated PKA was assumed to phosphorylate a substrate by converting ATP to ADP; and 3) the luciferase reaction was expected to catalyze a reaction where all remaining ATP was quantified as light. Although these three reactions were in excess and assumed to convert
all the remaining cAMP into a luminescence signal, in reality, equilibria exists. A more ideal assay would have fewer reactions between the quantification of the PDE3A hydrolysis reaction and the measure of output.

A third limitation of the PDE3A activity assay was that it was an endpoint assay. This means that the assay was terminated at different time points by saturating the PDE3A with inhibitor IBMX, and only after the PDE3A was no longer active, the quantification of the progress of the reaction was measured. Kinetic experiments are more commonly performed using real-time assays, where the reaction progress can be collected from one sample continuously over time. The real-time assay eliminates unnecessary sample variation and monitors the reaction trajectory over time. A real-time assay\textsuperscript{29} has been developed for the purpose of measuring the kinetic parameters of phosphodiesterases and could be used for future kinetic characterization of this system. Our purpose was to understand the mechanism of inhibition, and although trends were comparable between Compound 1B and competitive inhibitors, confirmation of competitive inhibition using kinetic parameters of a constant $V_{\text{max}}$ and increasing $K_m$ as inhibitor concentration increased, would be ideal using this real-time kinetic assay. The real-time assay determines the initial rates of cAMP hydrolysis by purified PDE protein by coupling the product 5'-AMP to the oxidation of NADH using three enzymes, adenylate kinase, pyruvate kinase, and lactate dehydrogenase. The spectrophotometric readout of product formation of the PDE hydrolysis reaction provides a continuous and therefore more accurate way to visualize the kinetic parameters of the reaction versus the readout of cAMP remaining from the PDE3A activity assay used in this study.
In our assays, the catalytic domain of PDE3A was used since the full-length PDE3A was not suitable for the activity assay due to significant instability after freeze-thaw. This shortened form of PDE3A protein may have masked informative results because previous studies suggest that the affinity of inhibitors for the catalytic site may depend on the presence of regulatory domains outside the catalytic domain of the PDE\textsuperscript{29}. For example, it was found that a PDE4D inhibitor, Rolipram, was 50X more potent at inhibiting the full-length protein than the catalytic domain. Other studies have suggested that cell lines expressing specific isoforms of PDE4D are more sensitive to inhibitors than other cell lines expressing another isoform\textsuperscript{30}. It has been proposed that regulatory domains finely control the activity of PDE4 by exposing or blocking cAMP from entering the catalytic site\textsuperscript{29}. These conformations are controlled by phosphorylation of the regulatory domains or the presence of partner proteins. The structural changes of the protein could be important for PDE3 as well because of the homology between PDE3 and PDE4 and because of the structural similarity across PDE inhibitors. Conformational changes in regulatory domains of PDE3A induced by inhibitors binding to the catalytic site could be responsible for selective toxicity in cell lines.

These findings of differential inhibition emphasize the need for understanding the isoforms present in the sensitive and nonsensitive cell lines to Compound 1B before we can elucidate the mechanism of selective toxicity. Applying these findings to our model, quantification of isoforms present in cell lines sensitive or nonsensitive to Compound 1B could reveal a correlation between specific isoform expression and sensitivity. Differential expression of isoforms with unique regulatory domains present could effect the regulation of the activity of the protein aside from the cAMP hydrolysis activity, such
as another interaction of PDE3A not yet characterized. The presence or absence of isoforms with the NHR1 and NHR2 regulatory domains could affect the binding of Compound 1B, and the ability to induce cell death aside from the cAMP hydrolysis function of the protein. The potency of Compound 1B in cell lines, in contrast to inactive analogs could be explained by the lack of favorable interactions directly between the compound and a regulatory domain or by the ability of the compound to induce an allosteric change elsewhere in PDE3A. Further inhibitor studies with various isoforms of the protein can be tested to determine if this relationship is true. These experiments would be performed in cell lines since cellular components necessary for phosphorylation or potential interactions with PDE3A would be relied on to perhaps see the functional effect (with lethality as output) of the binding of Compound 1B. Furthermore, mutational studies with a regulatory region believed to enhance the binding of an inhibitor could be performed to validate these interactions. Thermal denaturation assays could reveal further information of conformational differences between complexes of PDE3A/Compound 1B and PDE3A/non-lethal inhibitor. The isoform composition of cancer cell lines in conjunction with further structural insight on the PDE3A/compound complexes formed will reveal if sensitivity and isoform are correlated, implying that a regulatory mechanism of PDE3A may be involved in this phenotype of selective toxicity.

Similar findings to this study of the differential efficacy of PDE3 inhibitors have been reported involving megakaryocyte differentiation. Megakaryocytes are large cells in the bone marrow responsible for the production of platelets. A PDE3 inhibitor, Anagrelide, has been FDA approved to treat essential thrombocytosis, or high platelet count, by reducing megakaryocyte differentiation. However, the mechanism by which
Anagrelide affects megakaryocytes is not well understood\textsuperscript{31}. Although Anagrelide is a potent inhibitor of the cAMP hydrolysis function of PDE3, this function is not believed to cause the inhibition of megakaryocyte differentiation. Ahluwalia \textit{et al.} reported that not all PDE3 inhibitors induced the differential inhibition of megakaryocyte differentiation; specifically, Anagrelide, but not an equipotent PDE3 inhibitor Cilostamide, suppressed megakaryocyte development by reducing the expression of transcription factors GATA-1 and FOG-1\textsuperscript{32}. Because of the differential effect of PDE3 inhibitors, the authors concluded that a mechanism other than PDE3 inhibition was causing their phenotype. Wang \textit{et al.} concluded that Anagrelide inhibits megakaryocyte differentiation in a PDE3-independent manner\textsuperscript{33}. Both studies prematurely concluded that this phenotype is PDE3-independent. Their conclusions would have been convincing if they knocked-down PDE3A and cells treated with Anagrelide still inhibited megakaryocyte differentiation, while those incubated with Cilostamide did not. Otherwise, they cannot conclude solely on the discrimination of inhibitors that their phenotypic effect is PDE3-independent.

Despite these premature conclusions, their findings of inhibitor discrimination align with the findings we report. Although our lethality phenotype is observed in selected cancer cell lines, and inhibition of differentiation is observed in megakaryocytes, in both systems select PDE3 inhibitors induce the phenotypic effect while others do not. In our cancer cell line system, Angrelide induces the lethality phenotype (Hela EC\textsubscript{50} = 7.6-9.6 nM), while Cilostamide does not (Hela EC\textsubscript{50} = >10,000 nM). But unlike the previous studies on megakaryocyte differentiation, we are confident that our mechanism is PDE3-dependent because when PDE3A was knocked-out in sensitive cancer cell lines, Compound 1B no longer reduced viability (Luc de Waal, Unpublished Data, 2014). It is
conceivable that these systems are similar in the sense that the Anagrelide or Compound 1B/PDE3A complex is involved in a related pathway that induces these observed phenotypes. We have shown that very similar small molecules to Compound 1B will also bind to the catalytic domain of PDE3A, but do not induce the interaction necessary to cause the observed phenotype of cell death. This novel role of the inhibitor/PDE3A complex in a pathway related to lethality and perhaps megakaryocyte differentiation is outside the realm of inhibiting cAMP hydrolysis, and has not yet been determined in either system.

Although Compound 1B inhibits cAMP hydrolysis, this function alone is not sufficient for the lethality phenotype. The suggested novel role of PDE3A could be induced by a conformational change that occurs when Compound 1B is bound. This conformational change could prevent or enhance the ability of PDE3A to be phosphorylated, differentially localized, or capable of binding to a novel partner – a change that induces cancer cell death. One approach to uncover the implication of this complex is to perform an interaction assay in the presence of Compound 1B or a non-lethal competitive inhibitor. A Stable Isotope Labeling by Amino acids in Cell culture (SILAC) assay will be performed to co-precipitate interacting proteins with PDE3A incubated with Compound 1B and compare those proteins to the interactome in the presence of a non-lethal inhibitor. This quantitative proteomic approach incorporates heavy or light non-radioactive isotope-containing amino acids into newly synthesized proteins. For example a sensitive cell line incubated with Compound 1B could have heavy isotopes incorporated into synthesized proteins, while the same cell line incubated with a non-lethal inhibitor could have light isotopes incorporated. The isotopes allow for
the two protein populations to still be distinguishable after mixing the populations and co-precipitating with PDE3A at once to avoid variation in the handling of samples. The proteins and their abundances are identified by mass spectrometry and the differences in the interactomes of PDE3A can therefore be identified. These differences could reveal co-precipitating proteins from a novel biological interaction involving PDE3A, which is responsible for the cell lethality phenotype we observed.

The results of this study embody a close analysis of the interaction of a small molecule probe, emerging from a screen, and its target. Our study provides insight into the function of this probe, but the mechanism responsible for lethality has yet to be discovered. We assert that competitive binding of Compound 1B to PDE3A is important to conferring the signal for cell death in sensitive cancer cells, but further investigation is necessary to reveal the mechanism of this phenomenon in order to determine if treatment with Compound 1B or another therapy is a feasible therapeutic approach. As chemical screens are increasingly common to probe a biological question, functional follow-up is crucial to potentially translate experimental findings into therapeutics. Although the probes themselves may not be applied clinically, scientists will use them to provide insight in the novel pathways related to the biological phenotype. The current paradigm of drug development is often target driven, based on designing a compound to bind to a target, but far too often, the desired biological phenotype based on this interaction is difficult to achieve. The approach of selecting a probe from a phenotypic screen presents the challenge of uncovering the mechanism of action of the compound, but ensures that the compound induces the desired biological phenotype – a characteristic crucial for therapeutic development.
REFERENCES


