The Development of Phosphodiesterase 4D Inhibitors with 3D Printing and Molecular Visualization Software for the Treatment of Acrodysostosis

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Part 1: My Story

In some ways I think my Intel project brought together many of my high school career's loose ends. Everything seems to have lead toward this project. Freshman year of high school I had my first biology class, and became instantly fascinated with macromolecules like DNA and proteins. I have loved computer science since middle school, and in high school I programmed in Java for my school's FIRST Robotics team. I'll admit I hated math in elementary school, but once I started taking accelerated classes I enjoyed the challenge. By the time I started this project I had taken AP Calculus and AP Statistics, both of which were incredibly useful. Soon math, biology, and computer science began overlapping. Math is inevitably an integral part of computer science. Statistics was frequently vital to my biology labs. In Science Olympiad I used a program called JMOL to view macromolecules through Java code. For my Girl Scout Gold Award I taught the programming language Python to middle school students. This made it easy to later learn the Python version of JMOL, PyMOL.

I definitely would not (and could not) have done this project without the help of my mentor, Dr. Gurney. Dr. Gurney is a family friend and CEO of the biotech company, Tetra. He taught me the basics of structure based drug design, gave me a copy of the program PyMOL, and helped me with the chemistry aspect of this project. He encouraged me to enter my work in the Intel Science Talent Search, showed me how to perform essential lab procedures, and allowed me to use his lab space at Tetra. But others helped me too. Many of my high school teachers supported me, proof read my writing, and gave me their valuable opinions.

So my first suggestion for any student looking to undertake a science / math project is to reach out for help. Few great things can be accomplished alone. Secondly, pursue a subject you are passionate about. Sometimes the going gets difficult: sometimes the data doesn't line up, or the experiment was flawed, or you realize a simple mistake after days of work. You will only make it if you love what you're doing. Lastly: have fun.

Part 2: The Science

Introduction

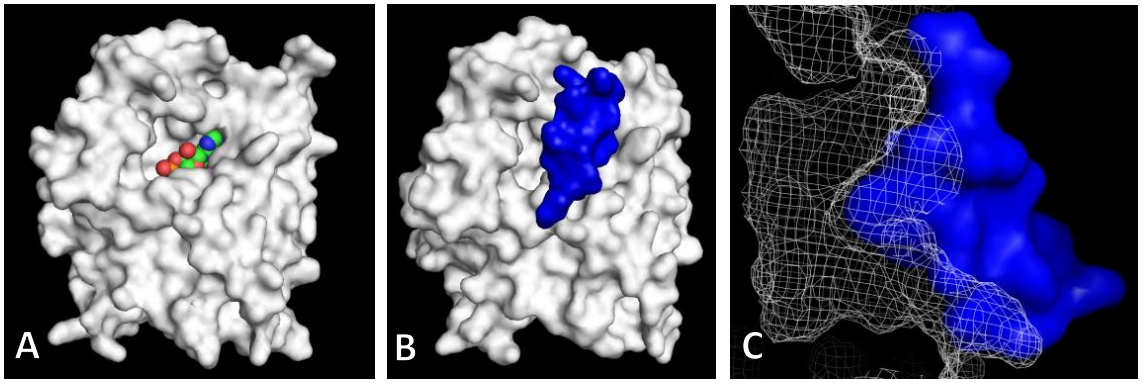
Although scientists do not yet fully understand how memories are formed, a protein called phosphodiesterase 4D (PDE4D) is clearly involved. Some children are born with mutated, damaged PDE4D, which results in a genetic condition called acrodysostosis. Kids with acrodysostosis typically have learning disabilities as well as short fingers, short toes, narrow faces, and short height. Currently there is no treatment for acrodysostosis, but this research shows it may be possible to use a small molecule to help mutated PDE4D and treat acrodysostosis. These small molecules may also treat Alzheimer's dementia, schizophrenia, depression, and Huntington's disease[3].

A Closer Look at Phosphodiesterase 4D

PDE4D is an enzyme that catalyzes the destruction of cyclic adenosine monophosphate (cAMP), a critical molecule for communication between cells. PDE4D contains two **U**pstream **C**onserved **R**egions of amino acid sequence, known as UCR1 and UCR2[3]. PDE4D contains a pocket in its surface, called the active site (fig 1A). This is where cAMP is broken down. UCR2, a short piece of protein, acts like a door. It swings open and closed to cover the active site. When it is closed, cAMP cannot reach the active site, and thus cannot be destroyed (fig 1B)[3]. When UCR2 sits in the closed position, a pocket remains between UCR2 and the catalytic domain (fig 1C). Designing a molecule that would complement the shape of this

pocket was critical to the drug discovery process.

Figure 1 Virtual representations of PDE4D from the molecular visualization software PyMOL (table 1).
(a) PDE4D catalytic domain (white surface), with cAMP (spheres) bound to active site.
(b) UCR2 (blue surface) in the closed conformation, covering the active site in the PDE4D catalytic domain (white surface).
(c) Cut-away view of PDE4D with UCR2 (blue surface) in the closed conformation. The pocket surface is displayed as a white mesh.



The Basis of Phosphodiesterase 4D Allosteric Inhibitors

When UCR2 closes over PDE4D's active site, a cavity remains between the two (fig. 1C). However, UCR2 does not fit very well. Molecules stick together better if their shapes are complementary. An allosteric inhibitor is a small molecule that helps UCR2 to fit on the active site by filling the cavity between them. An ideal PDE4D allosteric inhibitor fits perfectly matches the shape of the cavity.

The purpose of a PDE4D allosteric inhibitor is to secure UCR2 in the closed position, and thus prevent cAMP destruction. When UCR2 is open, the active site of PDE4D is exposed, and an inhibitor may enter the active site pocket. After UCR2 closes, cAMP can no longer reach the active site, and thus can no longer be destroyed.[3]

Shape is a critical part of drug design, but a successful drug must also meet strict chemical requirements. To slip into the bloodstream, an inhibitor must first dissolve in the stomach's watery environment. However, it must also be somewhat oily to diffuse through cells membranes. To monitor this balance between lipid and water solubility, I used the program ACD Chemsketch to calculate the partition constant (log *p*) of target molecules[10,11]. For a drug, this value ideally falls between three and

six[3]. The size of the compound must also fall within a narrow range. While binding affinity increases with the number of nonhydrogen atoms (plateauing at approximately 15 atoms)[6], the molecular mass must be less than 450 g/mol for the drug to avoid being filtered by the kidneys. The chemical properties of PDE4D inhibitors are not the focus of this research, but they do impose harsh constraints when optimizing a drug's molecular shape.

Developing a PDE4D Inhibitor

Through several rounds of structure based drug design I generated several molecules that could potentially work as PDE4D inhibitors. Iterative drug design works by designing only a few molecules, examining them closely, then designing a few more based on what was successful in previous rounds. Most drug development companies synthesize all the drugs and test in the lab. As this is very expensive and out of reach for high school student, I used software to simulate the interaction between each drug and PDE4D. Using PyMOL I was able to get a rough look at how well the drug fit into the active site-UCR2 cavity. Also, in a novel procedure, I used 3D printing to model PDE4D, UCR2, and drugs so that the structure could be more closely examined. This process is diagramed in Figure 2, and the complete list of considered drugs is shown in Table 1. In the end, Target 3 (Fig. 3), the molecule with the greatest binding affinity (best fit) was synthesized.

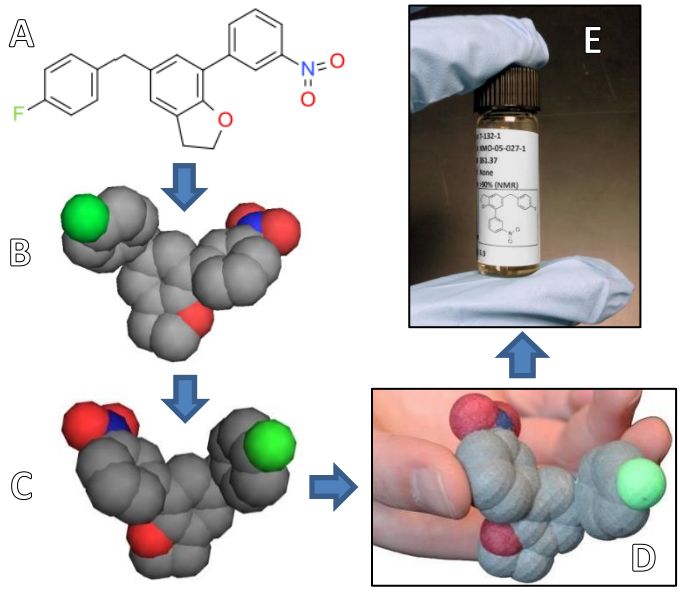


Figure 2 An overview the development of PDE4D inhibitor Target 3
A) A sketch of Target 3 in ACD Chemsketch
B) Target 3 in an energy minimized state calculated by CORINA, viewed in PyMOL
C) Target 3 positioned in ideal docking conformation by Autodock Vina, viewed with PyMOL
D) A physical model of Target 3, produced with 3D printing
E) A vial of synthesized Target 3

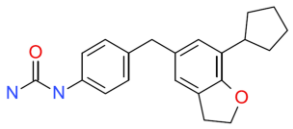
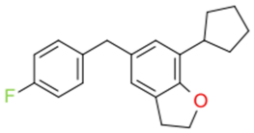
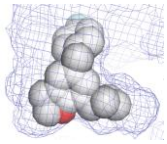
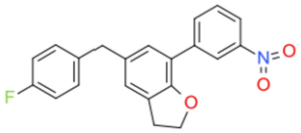
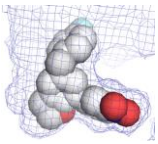
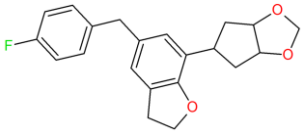
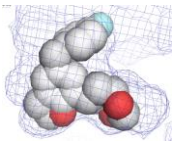
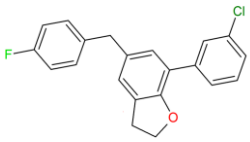
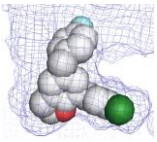
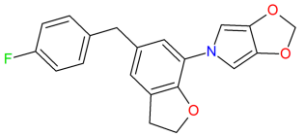
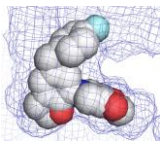
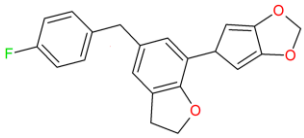
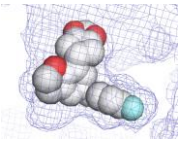
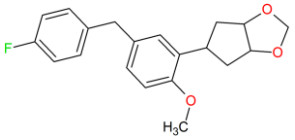
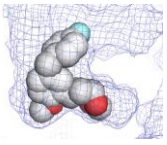
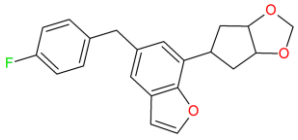
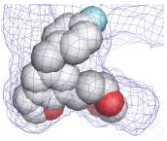
Inhibitor	Structure	Binding Affinity (kCal / mol)	Image of protein-drug complex in PyMOL
Target 1		N/A	N/A
Target 2		-11.5	
Target 3		-12.1	
Target 4		-10.4	
Target 5		-11.9	
Target 6		-10.4	
Target 7		-11.1	
Target 8		-10.2	
Target 9		-11.1	

Table 1 A summary of all PDE4D inhibitors designed during this project. Binding affinity and protein-drug complexes were calculated with Autodock Vina.

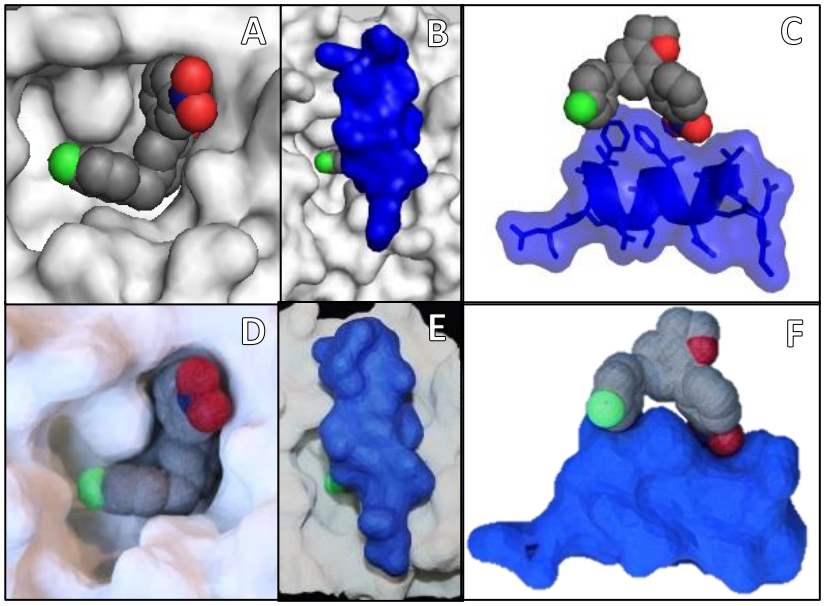


Figure 3 Target 3 (gray) as it binds with PDE4D (white) and UCR2 (blue). a – c) images from the molecular visualization software PyMOL. d – f) photographs of plastic molecular models I created with 3D printing.

Testing Target 3: Procedure

This process for quantitatively monitoring the rate of cyclic AMP hydrolysis by PDE4s was originally published by Burgin[3].

Each repetition of the biochemical assay tested three drugs. The first drug was my experimental inhibitor, Target 3. For comparison I also tested two well-studied PDE4D inhibitors, rolipram and D159687. They are highly potent reference drugs commonly used in PDE4D studies.[10,3]

This assay was performed in a 96 well plate, where the first two rows (A and B) were the negative and positive control, rows C and D contained rolipram, rows E and F used D159687, and the last two rows (G and H) contained my experimental drug, Target 3 (fig. 4a). In this manner, the assay was run in duplicate for each drug. The drugs were assessed at varying concentrations by using a fivefold serial dilution with DMSO. PDE4D (PDE4D-V5652) was added to all wells, followed by a solution containing adenylate kinase, pyruvate kinase, and lactate dehydrogenase. These three enzymes coupled the hydrolysis of cyclic AMP to the oxidation NADH. NADH oxidation decreases fluorescence, which allows for a quantitative measurement of the rate of cyclic AMP hydrolysis.

The final addition to the well plate was cAMP. Since cAMP in the presence of PDE4D

immediately begins to be destroyed, it was added to rows B-H at the last moment. To monitor each well's fluorescence over time, I used a Molecular Devices FMAX Fluorescence Microplate Reader (fig. 4b).[3]

Over several minutes the reader recorded each well's fluorescence on regular intervals. This data allowed the calculation of the rate of change in fluorescence for each well, from which the percent inhibition of PDE4D can be deduced with the following formula.

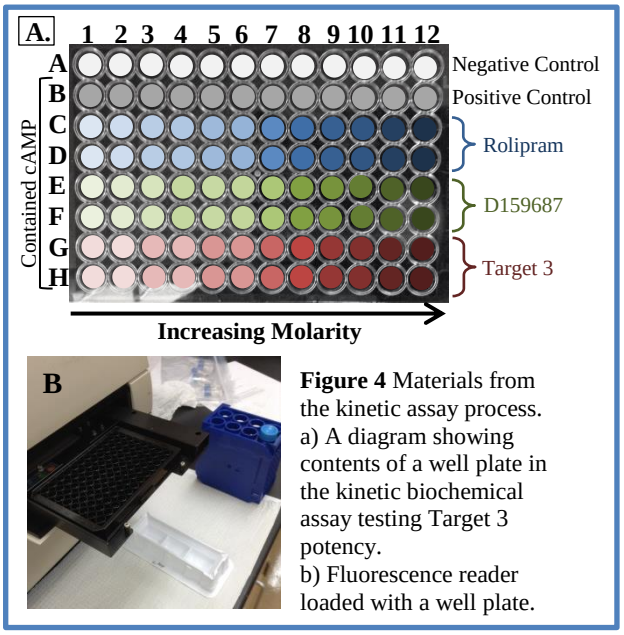


Figure 4 Materials from the kinetic assay process. a) A diagram showing contents of a well plate in the kinetic biochemical assay testing Target 3 potency. b) Fluorescence reader loaded with a well plate.

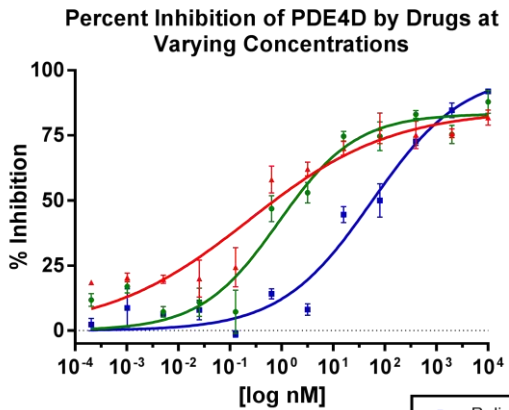
$$\% \text{ Inhibition} = 100*[(\text{AvgPos} - \text{sample}) / (\text{AvgPos} - \text{AvgNeg})]^{3}$$

Where AvgPos is the average rate of fluorescence change among the positive controls, AvgNeg is the average rate of fluorescence change among the negative controls, and sample is the rate of fluorescence change in the variable well.[3]

Testing Target 3: Results

The kinetic biochemical assay testing Target 3 as a PDE4D inhibitor relative to rolipram and D159687 was repeated twice. As each assay tested the ligands in duplicate, this yielded four total sets of data. I then graphed and analyzed this data using the statistical software Prism. The results are displayed in figure 5.

The data from the kinetic biochemical assay shows that Target 3 not only inhibits



	Rolipram	D159687	Target 3
EC50	56.24	0.8773	0.2337

Figure 5 The results from the kinetic biochemical assay testing Target 3 as a PDE4D inhibitor. This shows that Target 3 reaches 50% inhibition at a lower concentration than either reference compound.

PDE4D, but has a lower EC50 than both rolipram and D159687. The EC50 is what concentration of drug is required to decrease PDE4D activity by 50%. Rolipram and D159687 are extremely potent drugs—that Target 3's EC50 is one fourth of D159687's EC50 demonstrates that Target 3 is one of the most potent PDE4D inhibitors discovered to date.

A Look at Mutated PDE4D

From four independent studies[13-16], I compiled a list of PDE4D mutations resulting in acrodysostosis. The following experiment was performed to determine the activity of mutated PDE4D relative to healthy PDE4D. Determining the activity of mutated PDE4D was necessary so that later the effect of an inhibitor of mutated PDE4D could be seen.

I studied the activity of four different PDE4D enzymes: normal PDE4D (V5790), PDE4D with a mutation to simulate phosphorylation (V5652), and two PDE4Ds each with a different acrodysostosis mutation (V9020, and V9021) (fig. 6). For each enzyme I prepared 8 vials, each with a different concentration of the enzyme: 0, 0.5, 1, 2, 4, 6, 8, and 10 nM. I then transferred these solutions to a 96 well plate, as diagrammed (fig. 6). By transferring each solution to three separate wells the experiment was repeated in triplicate. In addition to the enzyme solution, I added a buffer solution to each well. As with the biochemical assay performed to examine Target 3's potency, the buffer solution contained adenylate kinase, pyruvate kinase, and lactate dehydrogenase. These enzymes coupled the hydrolysis of cyclic AMP to the oxidation of NADH. This meant that the more quickly PDE4D destroyed cAMP, the brighter the solution would glow. After adding cAMP, the fluorescence microplate reader was used to monitor the rate at which the glowing (fluorescence) decreased in each well, which indicated how quickly cAMP was destroyed.

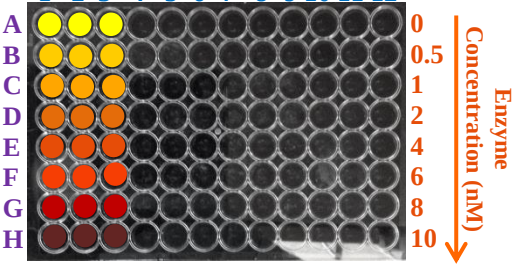


Figure 6 A diagram showing the setup of a 96 well plate for determining enzyme activity. The use of three columns allowed each enzyme to be tested in triplicate.

This experiment produced a measure of fluorescence over time for every well. For each

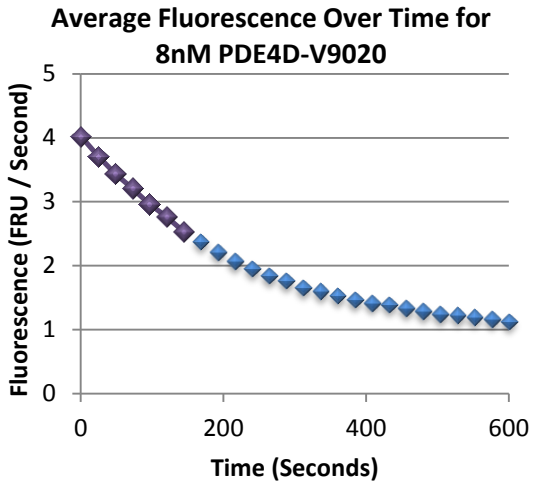


Figure 7 One of (4 enzymes * 8 concentrations) 32 graphs of fluorescence over time. The slope of the linear portion of the graph (in purple) is inversely related to the average activity of the enzyme at the given concentration.

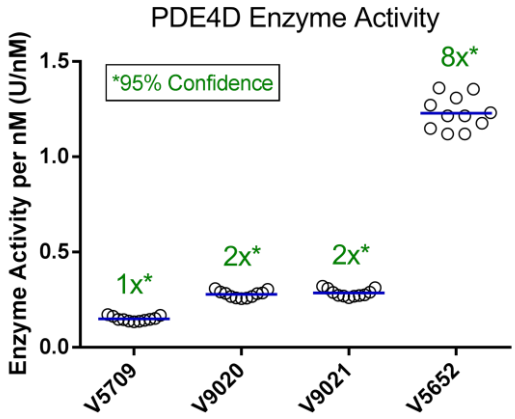


Figure 8 Data taken from the positive controls of the biochemical assays performed in the next section (Inhibition of Mutated PDE4D). Each point shows enzyme activity per nM for a well. Blue lines indicate average activity. One unit (U) represents -0.6 RFU/sec.
*Ratios show enzyme activity per nM relative to V5709. Ratios are confirmed with 95% confidence by a one way ANOVA test.

enzyme at each concentration, the three trials were averaged to produce an average fluorescence over time graph, such as in figure 7. This graph consisted of a linear portion followed by an exponential portion. The curved portion of the graph occurred when cAMP limited the rate of the reaction.

The slope of the linear portion of each graph was inversely related to the average activity of the enzyme. The greater the concentration of the enzyme, the faster cAMP was destroyed, and the more quickly cAMP was destroyed, the smaller (more negative) the slope of the linear portion of the fluorescence over time graph. By examining the relationship between concentration and rate in decrease of fluorescence for each enzyme, the relative activity of the enzymes could be determined.

Enzyme	Concentration (nM)
V5652	0.5
V5709-1	4
V9020	2
V9021	2

Table 2 The concentrations at which these four PDE4D enzymes have the same level of activity.

I wrote a Java program to do initial organization of the data, then used Prism and Excel to examine the outcome. For each enzyme, a concentration was chosen such that at that concentration each enzyme would have similar activity. The concentrations chosen for each enzyme are summarized in table

2. Data from the kinetic biochemical assay described below (under Inhibition of Mutated PDE4D) confirmed the chosen enzyme concentrations (figure 8).

Inhibition of Mutated PDE4D: Procedure

As the enzyme titrations suggests that acrodysostosis results from PDE4D hyperactivity, inhibiting PDE4D may serve as an acrodysostosis treatment. The following experiment was performed to determine how sensitive PDE4D with acrodysostosis mutations would be to PDE4D inhibitors.

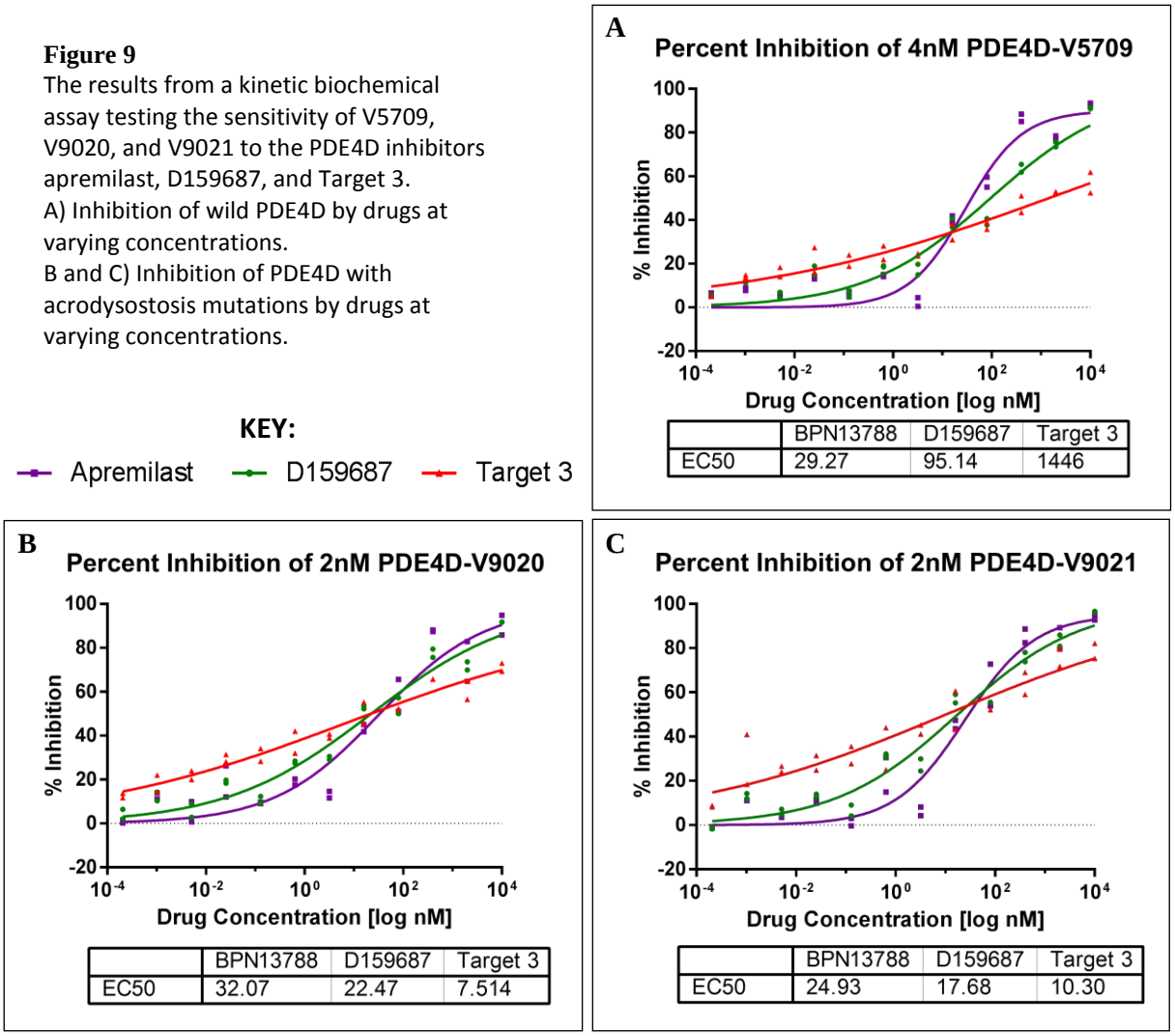
For this biochemical assay I worked with three PDE4D enzymes and three PDE4D inhibitors. The PDE4D enzymes were V5709-1, V9020, and V9021, prepared at the concentrations listed in Table 3. By preparing them at these concentrations, the three solutions had the same level of activity, within the margin of error (fig. 8). The three PDE4D inhibitors included D159687, apremilast, and Target 3. D159687 and apremilast are very potent PDE4D inhibitors commonly used for comparison in research.

Each of these drugs was tested as an inhibitor to each of these enzymes. In all other ways, this kinetic biochemical assay was like the kinetic biochemical assay used to determine Target 3's potency (described above.) A fivefold serial dilution was used to prepare each ligand over a range of concentrations. Two rows were left without drugs to serve as a positive and negative control. PDE4D and the adenylate kinase, pyruvate kinase, and lactate dehydrogenase solution were added to each well. After adding cAMP to all but the negative control, the fluorescence was monitored over time. The percent inhibition for each enzyme with each drug was calculated in the same manner as in the previous kinetic biochemical assay.

Inhibition of Mutated PDE4D: Results

Analysis of the data with Prism (fig. 9) revealed that PDE4D with acrodysostosis mutations could successfully be inhibited. All three drugs inhibited cAMP hydrolysis in V5709, V9020, and V9021, but for the acrodysostosis enzymes (V9020 and V9021), Target 3 had the lowest EC50.

Figure 9
The results from a kinetic biochemical assay testing the sensitivity of V5709, V9020, and V9021 to the PDE4D inhibitors apremilast, D159687, and Target 3.
A) Inhibition of wild PDE4D by drugs at varying concentrations.
B and C) Inhibition of PDE4D with acrodysostosis mutations by drugs at varying concentrations.



	BPN13788	D159687	Target 3
EC50	29.27	95.14	1446

	BPN13788	D159687	Target 3
EC50	32.07	22.47	7.514

	BPN13788	D159687	Target 3
EC50	24.93	17.68	10.30

Discussion and Conclusion

The success of Target 3 not only identifies a new family of PDE4D inhibitors, but lends credibility to the use of 3D printing as a tool in drug design. From the first successful models of UCR2 and D159153, it was clear that 3D printing could be powerfully helpful in understanding complex molecular structure. The physical models revealed that several assumptions about UCR2 / drug interaction were inaccurate. For example, until viewing the UCR2 and D159153 plastic models, my mentor had always pictured the UCR2-drug complex incorrectly (fig. 10). The 3D modeling enhanced qualitative analysis of the drug-protein complex, allowing me to more precisely craft a compound to

perfectly fit the pocket between UCR2 and the PDE4D catalytic domain. By examining the molecules with modeling and testing them with docking software, I performed several rounds of iterative structure based drug design without expensively synthesizing each compound. Only the most promising target was synthesized. The kinetic biochemical assay revealed this molecule, Target 3, to be one of the most potent PDE4D inhibitors discovered to date.

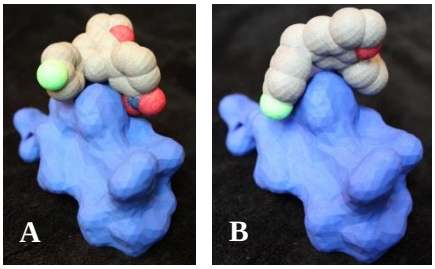


Figure 10 Plastic models of UCR2 (blue) and Target 3 (gray)
a) UCR2 and Target 3 in correct configuration
b) Approximately how my mentor had envisioned the UCR2-drug complex prior to seeing the 3D printed models.

The PDE4D titration showed that PDE4D with acrodysostosis mutations is approximately twice as active as normal PDE4D. This implies that acrodysostosis symptoms result from PDE4D hyperactivity. The data from the second kinetic biochemical assay revealed Target 3 to be a potent inhibitor of PDE4D enzymes with acrodysostosis mutations. By partially inhibiting hyperactive PDE4D, Target 3 has the potential to work as the first acrodysostosis treatment.

The importance of PDE4D inhibitors in memory and learning pathways makes Target 3 a potential treatment for Alzheimer's dementia, schizophrenia, depression, and Huntington's disease. More research in needed to investigate the possibility of using Target 3 is a treatment to these diseases. The results of this research show that the PDE4D inhibitor developed during this project has the potential to treat acrodysostosis. With early intervention, individuals diagnosed with acrodysostosis could possibly prevent late onset symptoms and some mental disability by taking Target 3.

References

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