THE SELECTIVE INHIBITION OF G PROTEIN-COUPLED RECEPTOR KINASES USING SMALL MOLECULE INHIBITORS

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Abstract

Expression of G protein-coupled receptor kinase 2 (GRK2) and the related protein GRK5 plays a vital role in the regulation of cardiac activity by inhibiting G protein-coupled receptor (GPCR) signaling in the heart. GPCRs such as the β_1-adrenergic receptor, which control the contractility of the heart, are dramatically down-regulated during heart failure, a consequence of overexpression of GRK2 and GRK5. Previous studies have indicated that the overexpression of GRK2 and GRK5 can lead to heart failure in mouse models. The goal of my first project was to identify small molecule inhibitors that selectively targeted GRK2 over other GRKs. In this study, we conducted screens at the Center for Chemical Genomics which identified a small molecule, paroxetine, which displaces an RNA aptamer bound to the active site of GRK2. Follow up analysis with kinase activity assays demonstrated selective inhibition of the protein against other GRK subclasses. This study was followed by a parallel project with the goal of identifying selective inhibitors of GRK5. In this project with GRK5, we started off by screening using a thermostabilization assay, which is more applicable to the screening of proteins that do not have a competitive binding molecule similar to GRK2's aptamer. Using similar techniques, a small molecule, amlexanox, was identified as a relatively selective and potent inhibitor of GRK5.

Introduction

G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest transmembrane receptor family in humans and a popular target in pharmacology, with around 50% of all medications acting on them[1]. As transmembrane proteins, they act to induce intracellular responses from extracellular signals through ligand binding. Also known as "serpentine receptors", these proteins consist of seven transmembrane domains and each receptor is associated with G proteins, which act as intermediaries between the stimulation of the receptor and the secondary messenger signaling cascade, leading to a variety of responses.

These receptors are responsible for the regulation of a number of universal physiological responses, which include visual and olfactory sensing, immune system and inflammation regulation, autonomic nervous system transmission, and cell density sensing. Consequently, misregulation of GPCR signaling has been connected to multiple conditions, which include diabetes, blindness, allergies, depression, cardiovascular deficiencies, and certain types of cancer[2].

G protein-coupled receptor kinases

One of the mechanisms by which these signaling cascades are regulated is through G protein-coupled receptor kinases (GRKs). In the event of stimulation of GPCRs, GRKs function to inhibit the signaling cascade by phosphorylating the serine or threonine residues on the intracellular loops and/or C-terminus of the active receptor, which in turn increases the binding of arrestins on the intracellular side of the protein[3]. Once bound, arrestin inhibits signaling from the receptor in multiple ways. Steric hindrance against the coupling interaction between the

receptor and the G protein allows for continuous ligand binding on the extacellular side of the receptor, but does not allow for the stimulation of the associated intracellular G protein. Arrestins also recruit other proteins through a scaffolding mechanism that inhibits the receptor through activation of the extracellular-signal regulated kinase (ERK) pathway or through the sequestration of the receptor via endocytosis into the cell. The endocytosis of the receptor allows for variable regulation, based on the type of desensitization required. For long-term exposure to a hormone, the receptors are only sequestered and dephosphorylated, allowing them to be re-integrated back onto the membrane, resensitizing the cell. In different circumstances, the receptor may be degraded via lysosomes and never return back to the cell surface[4]. All of these mechanisms effectively desensitize the receptor from extracellular ligand binding by uncoupling the receptor from its downstream components, thus preventing the over-activation of the secondary messenger cascade[5].

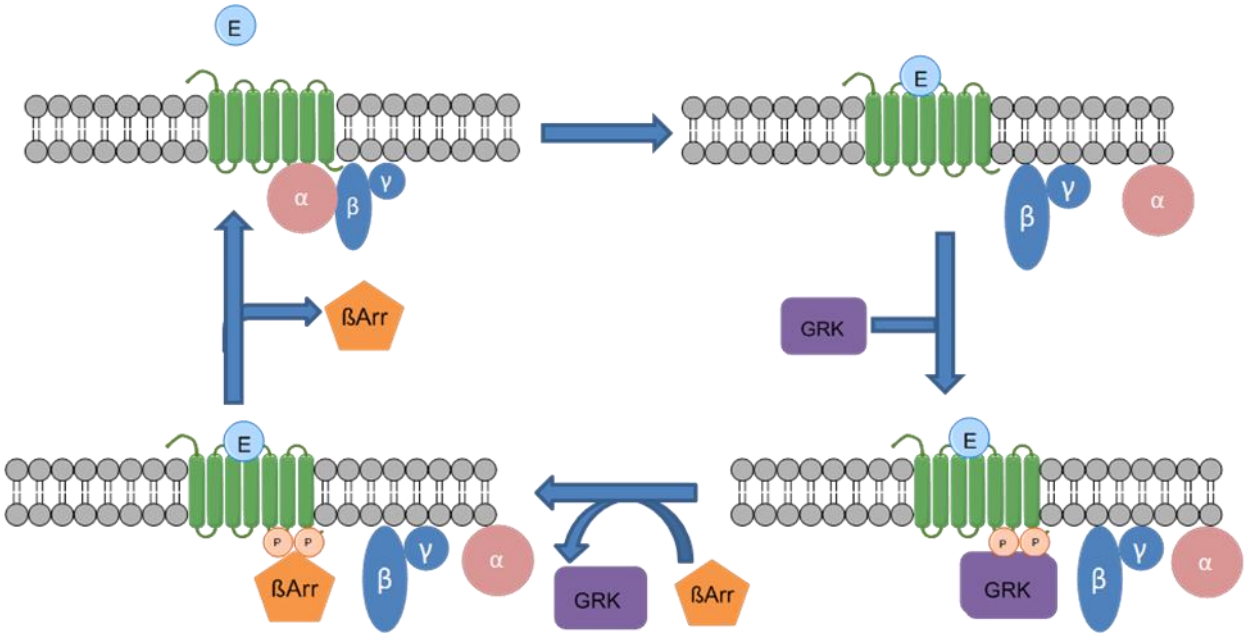


Figure 1. GRK regulation of GPCRs
A GPCR on the membrane is activated via a hormone (in this case epinephrine) or some other agonist and stimulates nucleotide exchange on the G protein alpha subunit (G_{α}) which activates the G protein. Once activated, the G_{α} and $G_{\beta\gamma}$ subunits dissociate from each other and activate downstream signaling cascades. GRK is then able to associate with and phosphorylate the active receptor. The phosphorylated carboxyl terminal binds to an arrestin, which

initiates the inactivation of the receptor. The receptor is later able to be reactivated once arrestin dissociates, and the GPCR again couples with the heterotrimeric G protein.

There are seven different GRKs (GRK1-GRK7) in humans, which are categorized into three different sub-families of GRKs based on structural homology and functional similarities[6]. The GRK1 sub-family, which is also known as the rhodopsin kinase family, consists of GRK1 and GRK7. These are mostly expressed in the retina and are mostly involved in dampening reactions to light in the retina. The GRK2 sub-family, which is also known as the β-adrenergic receptor kinase family, consists of GRK2 and GRK3. These are expressed in many different organs throughout the body such as in the heart, lungs, brain, and placenta, and function in a wide range of regulatory roles. GRK2 and GRK3 both require binding to membrane-associated $G_{\beta\gamma}$ for activity in living cells[7]. The final sub-family (GRK4) consists of GRK4, GRK5 and GRK6. These members are widely expressed, and are found in a variety of organs[8, 9, 10, 11]. GRK5 is considered the most widely expressed member of this subfamily. Unlike the GRK2 sub-family, this sub-family does not require the association of $G_{\beta\gamma}$ for activation[12].

GRKs and Cardiac Regulation

β-adrenergic receptors are GPCRs that respond to epinephrine to control the contractility of the heart to regulate the force at which the heart beats. In human myocardial cells, the β_1 and the β_2-adrenergic receptors are the main regulators of adrenergic effects through coupling with a specific G_α protein[13]. Increased stimulus via these catecholamines, such as epinephrine leads to tension and hyperactivity, as the sympathetic nervous system is forced to meet the energy needs associated with increased activity and physical exertion. Chronic stimulation of the β_1-adrenergic receptor in particular has been associated with negative effects on overall myocardial health[14].

Similarly, it has been shown that there is a strong correlation between decreased density and responsiveness of the β_1-adrenergic receptor and heart failure[15-19].

The expression of G protein-coupled receptor kinases 2, 3 and 5 (GRK2, GRK3 and GRK5, respectively) plays a vital role in the regulation of this cardiac activity, with GRK2 and GRK5 being the primary GRKs expressed in the myocardium[9, 12, 20]. In situations where the β-adrenergic receptors (β−ARs) are upregulated, the return to homeostasis via downregulation of the receptors is mediated by these GRKs. It is believed that the downregulation of β-AR signaling mediated by GRKs is a mechanism to protect against catecholamine cytotoxicity. Pathological downregulation of the receptor occurs in instances where there is chronic catecholamine release, such as during heart failure. The subsequent decrease in cardiac output from GRK signaling also leads to continued stimulation of the sympathetic nervous system, ultimately resulting in a feedback loop which leads to the dramatic down regulation of the receptor[21]. The over-expression of GRKs has been found to play a key role in conditions such as heart failure in numerous experimental models[18-20, 22-31] (Figure 2).

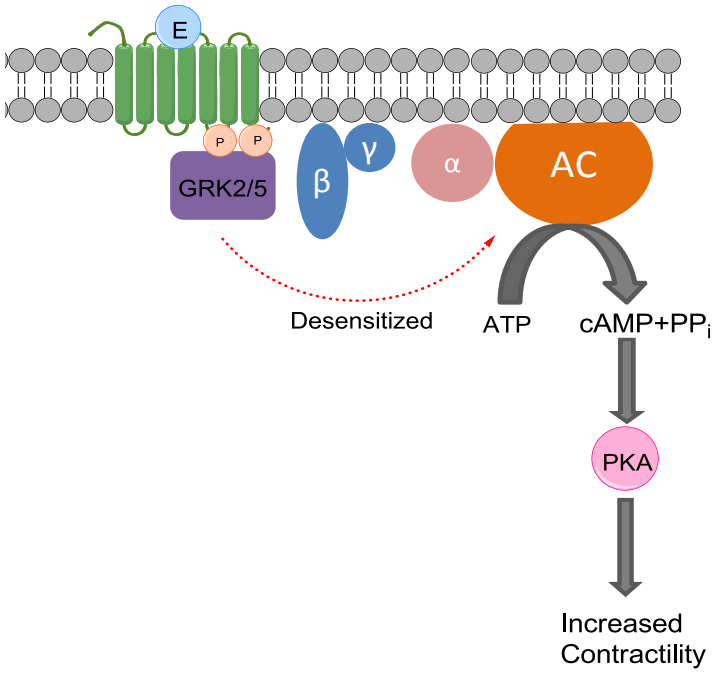


Figure 2. β-adrenergic receptor regulation by GRK2/5
The β-adrenergic receptor, which controls the contractility of the heart via stimulation of adenylyl cyclase and protein kinase A, is acted upon by catecholamines (CA) such as epinephrine. Binding of epinephrine stimulates the β-adrenergic receptor, causing an increase in contractility. Prolonged CA signaling leads to increased production of GRK2/5, which phosphorylate the receptor and triggers its desensitization. The up-regulation of GRK2/5 has the possibility of leading to heart failure by severely down-regulating this receptor, compromising cardiac function, and making the heart incapable of pumping blood throughout the body.

G protein-coupled receptor kinase 2

The protein GRK2 has been shown to be connected to hypertension[32], cardiac hypertrophy[33], and myocardial ischemia[22]. An increase in GRK2 expression has also been shown to be strongly correlated to the onset of heart failure, suggesting it as a potential therapeutic target[23, 28, 34]. GRK2, when active, phosphorylates the β_1-adrenergic receptor (β_1-AR) and, through the previously discussed mechanisms, decreases the sensitivity to agonist stimulation thereby counteracting the induction of hypertension, thus decreasing the contractility of the heartbeat. When GRK2 is overexpressed, the β_1-AR is severely downregulated, sometimes resulting in heart failure. In vivo studies have shown that inhibition of GRK2 has prevented heart failure in mouse models, making it a prime therapeutic target. The identification of small molecule inhibitors of GRK2 would be a step towards a new treatment for heart failure. Rather than aiming at the receptor, which has been targeted by other beta-blocking drugs, we would be targeting a different part of the pathway that underlies many aspects of heart failure. Based on this premise, the overall goal of the first project is to find small molecules that selectively inhibit GRK2, thus preventing the effects of its over-expression.

G protein-coupled receptor kinase 5

Similar to GRK2, GRK5 plays a significant role in regulating cardiac contractility by inhibiting the β_2-adrenergic receptor (β_2-AR). Overexpression of GRK5 has been shown to play

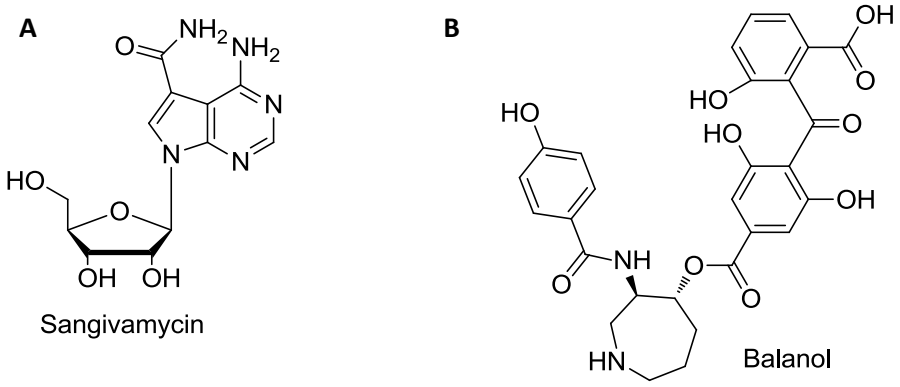
a role in heart failure in mouse models[5]. GRK5's nuclear localization in myocardial cells and more gradual changes in expression during heart failure make it unique in terms of its overall contribution to disease, and it has been suggested that GRK5 directly affects hypertrophic gene transcription[35]. The phosphorylation of the β_2-AR by GRK5 desensitizes the receptor in a manner similar to GRK2's desensitization of the β_1-AR. GRK5 overexpression as an overcompensating response to the stimulation of the β_2-AR analogously leads to the decrease in contractility demonstrated with GRK2 [36-38]. Consequently, the inhibition of GRK5 expression in these instances has demonstrated to be protective against heart failure[39]. Whereas GRK5 inhibition correlates strongly with cardiac protection, no bioavailable inhibitors have been discovered for GRK5. The goal of the second project was to screen for selective small molecule inhibitors of GRK5 using procedures similar to those used in the screening of GRK2 inhibitors.

The discovery of a specific inhibitor of GRK5 serves multiple purposes. A bioavailable inhibitor would be a novel therapeutic approach towards protecting against heart failure. As of yet, no safe and marketable medications targeting this particular protein have been reported. With the knowledge of its therapeutic potential, a bioavailable inhibitor could open up new avenues of cardiovascular treatment. The discovery of a new binding molecule to the protein GRK5 could also aid in the elucidation of the protein's structure. As of yet, no structure has been found for GRK5, making a more stable tertiary conformation desirable for further studies with the protein. Obtaining clues about the structure of the protein could potentially serve as a starting point for subsequent experiments and aid in the design of a new and more specific inhibitor.

Previously Studied GRK Inhibitors

Other GRK inhibitors have been described, but none have ever been selected for clinical trials due to a lack of selectivity and/or oral bioavailability. These known inhibitors include sangivamycin, balanol, the Takeda compounds 101 and 103A, and the RNA aptamer C13. These previously studied GRK inhibitors are used in the project as controls for our own screening efforts.

Sangivamycin, an adenosine analog (Figure 3A) that mimics the substrate ATP, which is used for phosphorylation of activated GPCRs[40]. This allows sangivamycin to bind in the active site of GRK2 and inhibit kinase activity. Balanol is a natural product that acts as a nonselective AGC kinase inhibitor (Figure 3B). It has also been shown to selectively inhibit certain GRK subfamilies by binding to the kinase site. Balanol induces a more closed, but not completely closed, active site on the protein, forming a unique and inactive conformation[41, 42]. Takeda Pharmaceuticals, Inc. reported in a patent the development of potent and selective inhibitors of the GRK2 subfamily that were proven to bind in the active site of the enzyme[42, 43]. These compounds, 101 and 103A, never made it to clinical trials, presumably due to their low bioavailability (Figures 3C and 3D).



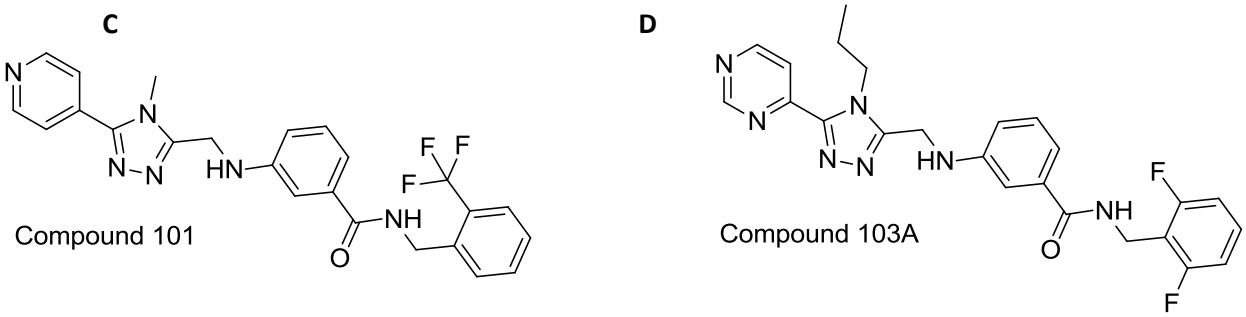


Figure 3. Known Inhibitors of GRK2
(A) Sangivamycin, (B) Balanol, (C) Takeda Compound 101, and (D) Takeda Compound 103A. These inhibitors were used as controls for various stages of the study.

In a previous study, an RNA aptamer called C13 was found to selectively inhibit GRK2 with a K_d= 1.5 nM. The aptamer forms contacts both inside and around the active site[44] (Figure 4A). Crystallographic studies done using a truncated form of the aptamer (C13.28) demonstrated that the binding of this aptamer to GRK2 induces a novel conformation of the protein, suggesting that compounds that competitively displace this aptamer might also induce a unique and stable conformation.

It should be noted that RNA aptamers are not considered viable therapeutics for pharmacological study with regards to oral therapy. It has been shown that these polymers have many of the properties characteristic of potential intracellular inhibitors such as selective interaction, relatively high affinities for protein binding, and inhibition of catalytic activity on the target protein[45-47]. The problem lies in their bioavailability, as there is no technology as of yet to facilitate their transmembrane delivery into the target cells. It is thus believed that an ideal solution for both pharmacological study and medical application would be in the area of small molecule inhibitors, which are more bioavailable than their RNA counterparts and able to more selectively inhibit certain parts of a protein rather than affecting the entire structure. For the first

project, we used the C13 aptamer to perform an aptamer-displacement assay to identify potential small molecule inhibitors[48].

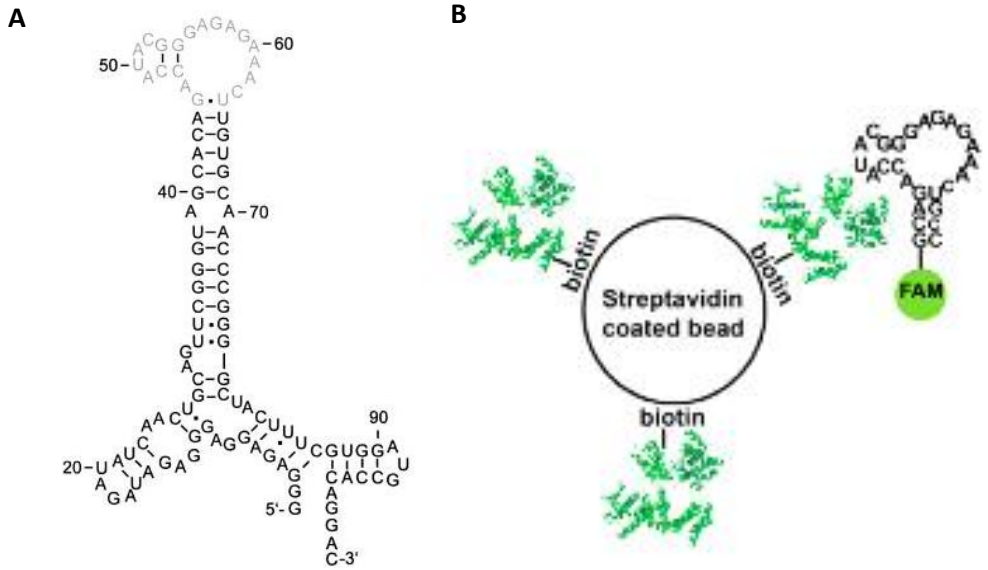


Figure 4. RNA aptamer C13 interacts with GRK2
(A) The aptamer C13 was used in this experiment as a competitive and selective inhibitor for GRK2[44]. (B) For flow cytometry protein interaction assay (FCPIA) testing, C13.28-FAM, was used in competition with the various compounds. The biotinylated protein was attached to streptavidin beads for reading via flow cytometer.[49]

Methods

Flow Cytometry Protein Interaction Assay (FCPIA).

RNA aptamers have been used to identify other specific small molecule inhibitors in aptamer-displacement assays[48]. With the aptamer's selectivity and high affinity for binding to GRK2, the C13 aptamer is much more likely to be out competed for binding to GRK2 only by other selective and high affinity compounds[44]. In this assay, we used the C13.28 aptamer, a truncated form which has a shorter stem loop structure, to test for competitive binding of small molecules to GRK2.

For our primary screening of compounds, we used a Flow Cytometry Protein Interaction Assay (FCPIA), a high-throughput bead-based flow cytometry assay that had previously been shown to successfully measure protein-protein interactions with GRK2[50]. GRK2 was

biotinylated using an amine-reactive probe (Sigma) and purified over a gel-filtration column. The biotinylated protein was attached to streptavidin-coated polystyrene beads (Spherotech) as shown in Figure 4B, forming a covalent-like interaction to ensure a high signal. This was done by incubating beads in 18 μM protein for at least 30 min. Extraneous unbound protein was taken out of solution so as not to cause background interference in the assay. The beads were spun down and resuspended in flow buffer (20 mM HEPES pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM CHAPS, 0.2% lubrol, and 2 mM DTT) at 12 rcf for 60 seconds. The supernatant was removed, beads were resuspended in 1 mL of flow buffer, and the process was repeated three times. The final concentration of GRK2 used in these assays was 10 nM for each sample tested.

The bound GRK2 was then incubated with the test compounds. The compounds were dissolved in pure DMSO and stored on stock plates at room temperature at a concentration of 1 mM. Compounds were placed with buffer on 384-well plates (Corning) at a final concentration of 10 μM with 1% DMSO using a Beckman Biomek FX robot (Beckman Coulter). GRK2 and the test compounds were then left to incubate for thirty minutes on the plate.

The final step involved the binding of the RNA aptamer to the protein. A truncated and fluorescein-labeled form of the aptamer (C13.28-FAM, Figure 4B) was used for the assay to test for competitive binding against the test compounds. The labeled aptamer had been proven to be indefinitely stable when stored at -80˚C and stable for up to 5 days when kept on ice, dispelling any concerns that degradation could occur in the assay. To initiate interaction between the GRK2 and the aptamer, C13.28-FAM was plated with a final concentration of 2 nM and allowed to incubate for 1 hr.

Once the mixture had had time to reach equilibrium, the displacement of the C13.28-FAM and binding of the compounds to GRK2 was measured using an Accuri C6 Flow

Cytometer and analyzed using Accuri HyperCyt software. Each well was gated, and the amount of fluorescence was measured for each well. The fluorescent level indicated the amount of binding activity between the protein and the compound. With competitive binding, we would expect that the protein would displace C13.28-FAM, thus decreasing the fluorescence of the protein on the beads. Therefore, the less fluorescent signal read by the flow cytometer, the greater the amount of binding implied between the compound and the protein.

Each plate had positive and negative controls in order to gauge how much of an effect each compound had on signaling. The positive control consisted of blank beads, which gave off no fluorescent signal, representing a protein bound to a compound which fully competed against aptamer for binding. The negative control consisted of beads that were directly attached to C13.28-FAM-bound GRK2 in the presence of a DMSO control. This sample represented a compound that did not bind at all to the protein, failing to compete with the RNA aptamer. Based on these readings, we were able to determine the relative binding activity of each compound onto GRK2.

FCPIA, although extremely effective in its ability to test for competitive binding, can only be used to test for GRK2 inhibitors. This test was only effective against this particular member of the GRK family due to the fact that the RNA aptamer used for competitive analysis was specific for GRK2. Other RNA aptamers with specific binding for the other various GRK proteins have not been developed, and thus are not available for usage in competition assays.

Aptamer-Based FCPIA Control Assay

Because this assay was dependent on the change in fluorescence upon C13.28-FAM binding to the protein, these results were then tested against a second control screen. This

consisted of testing the selected compound hits against plates that contained streptavidin beads labeled only with biotinylated aptamer (b-C13.28-FAM) and no protein. Any compounds that interfered with the assay by binding to the aptamer and not to the protein or interfered directly with fluorescence readings were dismissed, and those hits that gave negative readings against the confirmation aptamer assay were followed up on.

FCPIA Dose Response Assay

In order to assess the strength of the interaction between the binding compounds and the protein (GRK2), a dose response was performed to test the binding activity of the compound at various concentrations. In these assays, the compound was diluted down the plate in a two-fold dilution series and incubated with a common concentration of GRK2 and C13.28-FAM. The concentrations used for dose response ranged from 200 μM to 50 μM. All preparations regarding the protein GRK2 and the aptamer did not change from the single-point protocol described above for the general screen.

The binding resulting from the various concentrations of inhibitor can be charted in a dose response curve which can be used to quantify the potency of the compound against the protein (Figure 5). This quantification is measured by determining the IC_{50} value, or the half-maximal inhibition concentration. The IC_{50} value is the concentration of the compound which decreases the activity of the protein to half of its native biochemical activity. This is based upon the controls in each plate that represent full activity (or no binding) and completely inhibited activity (100% binding). In FCPIA, the IC_{50} would represent half-maximal binding of the aptamer C13.28-FAM.

Control screens similar to those of the original, single-point FCPIA assays were also used here for determining compound-aptamer interaction. b-C13.28-FAM was attached to streptavidin beads and used in place of protein when incubated with the series of compound concentrations. This was used to determine the strength of the interaction between the compounds and the aptamer and compare it to the interaction between the compound and the protein.

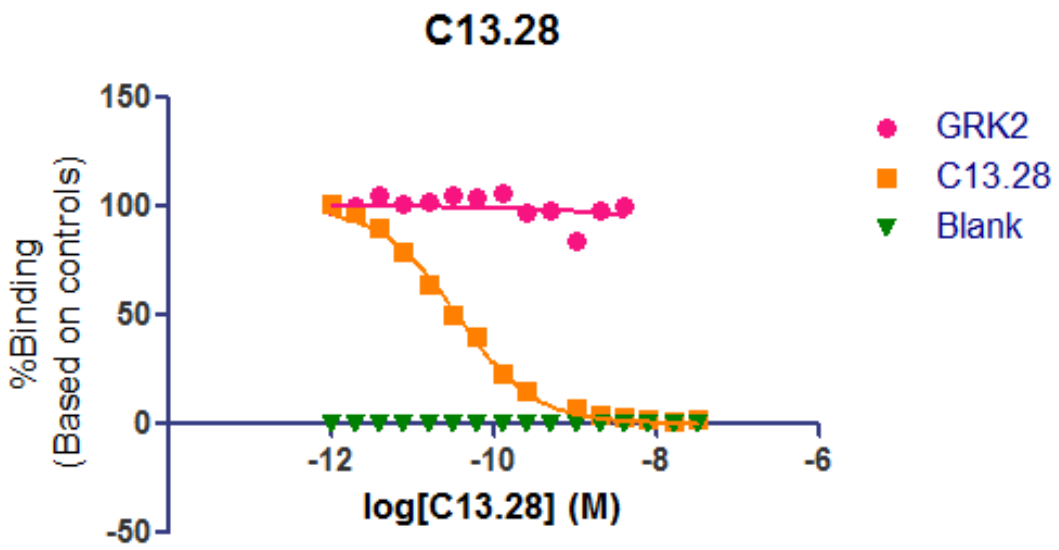


Figure 5. A typical normalized dose response curve obtained using FCPIA. This curve depicts the complete competition of unlabeled C13.28 aptamer against labeled C13.28-FAM for binding to GRK2.

Thermofluor Assay

This stability of a protein can be gauged by measuring its melting temperature (T_m), which is the temperature at which a protein population denatures. A protein-compound complex which forms a stabilizing interaction requires more heat to denature the protein, whereas a compound which destabilizes the protein will cause the protein to denature at a lower temperature. These interactions, as measured through shifts in melting temperature from pure protein to protein-compound interaction, strongly correlate with the level and nature of the

binding reactions that take place between the selected small molecules and the protein. The thermofluor assay is a test that determines the melting point of GRK2 while interacting with a compound by analyzing the changes in the state of the structure over a wide temperature range.

1-anilinonaphthalene-8-sulfonic acid (ANS) is a fluorescent compound that fluoresces at a wavelength of 380 nm[51] (Figure 6A). The fluorescence of ANS is quenched in aqueous solution, but increases in nonpolar environments. Using ANS (Sigma), we can observe the thermal denaturation of GRKs over our chosen range of temperatures. Once ANS interacts with the hydrophobic residues found at the interior of the protein structure, the fluorescence reading increases. The greater the fluorescence reading, the more exposed hydrophobic regions are interacting with ANS.

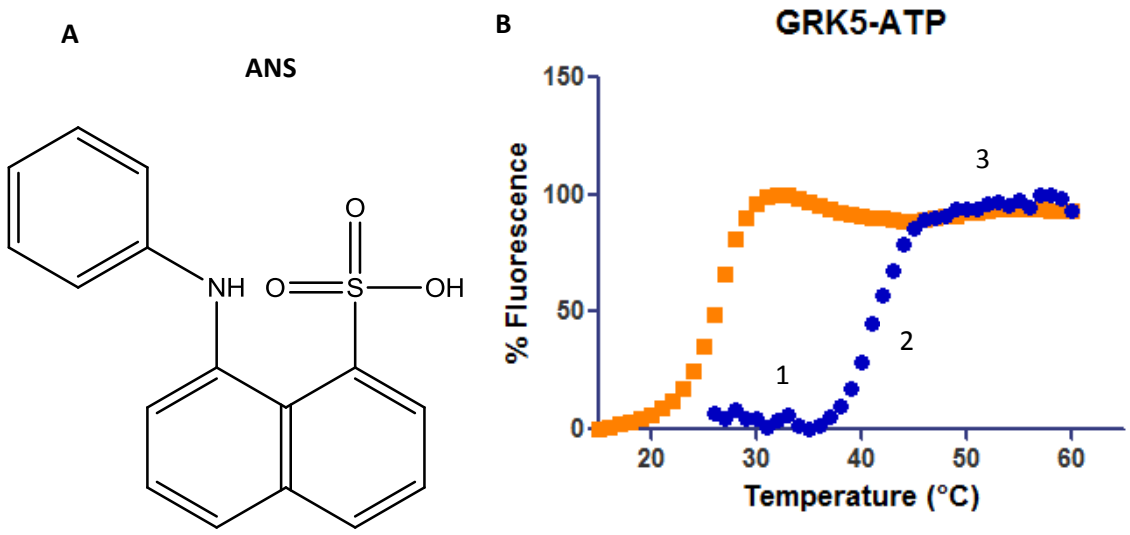


Figure 6.

(A) 1-anilinonaphthalene-8-sulfonic acid (ANS) only fluoresces in non-polar environments. In the thermofluor assay, this is used to measure the denaturation of the protein, as fluorescence can be used to measure the amount of interaction ANS has with the hydrophobic interior of the protein[51]. (B) As a control, ATP bound to GRK5 shows a significant increase in T_m with a ΔT_m of 15.5˚C. The graph for thermofluor takes on a characteristic sigmoidal curve with three distinct regions. These areas represent (1) a baseline of completely natured and stable protein-compound complex, (2) the increase in signal associated with the denaturation of the protein, and (3) the plateau indicating a completely denatured protein.

For this assay, compounds were set on a 384 well plate either by using the Beckman Biomek FX robot (Beckman Coulter) or by hand with a pipette at a volume of 0.2 μL. All of the compounds used were in solution with DMSO at varying concentrations based upon the compound. With a final volume of 5 μL, the concentration of the compound from the stock solution was diluted by ~25x, making the DMSO concentration negligible for each experiment. The compounds were plated with negative controls of DMSO alone.

The protein and ANS were placed into a single stock solution with 20 mM HEPES pH 7.0, 5 mM $MgCl_2$, 1 mM CHAPS, and 2 mM DTT, with a final concentration between 0.075 mg/mL-0.2 mg/mL for the GRKs and 100 μM for the ANS. 5 μL of the stock solution was added to each of the wells and spun down in a plate centrifuge for 1 min at 1000 rpm. After spinning the plate to ensure that the solution is at the bottom of the well, 2 μL of silicon oil (Aldrich) was added to the top of the well to ensure minimal evaporation during the course of the experiment and the plate was spun again. The protein-compound-ANS mixture was then incubated for 15-45 min, allowing the compound and protein to interact.

The plate was read using a ThermoFluor machine (Johnson & Johnson) over a temperature range appropriate for the protein being tested. GRK2 has a melting temperature of 36.6˚C, which is higher than that of the other GRKs. In contrast, GRK5 only has a melting temperature of 25.9˚C, while GRK1 has a melting temperature of ~22.3˚C. The ranges of temperatures tested in the thermofluor assay were changed accordingly. For GRK2, the temperature range generally used was 25˚C-70˚C, while GRK1 and GRK5 are tested at 15˚C-60˚C.

Over these ranges of temperatures, the graph that results from each sample takes on a characteristic curve that can be broken down into three sections (Figure 6B). The first section is the baseline which indicates a stable protein-compound equilibrium. At this point, the protein has not yet begun to denature and is still in its native conformation. The second part of the graph shows the steady increase in fluorescence signal which indicates the process of denaturation of the protein. As the protein continues to be denatured, the ANS has more access to the interior hydrophobic residues, resulting in this sudden increase in signal. The final part of the graph is the plateau indicating the completely denatured state of the protein. The graph as a whole is read as a sigmoidal curve, with the point of inflection used as the melting temperature of the protein (Figure 6B).

Two different settings for the reading of the plate were used for various experiments. Continuous Ramp was used for the GRK2 experiments, where the machine gradually increased the temperature by 1˚C over a period of 1 min and then took a reading for each set degree. The problem that came from this was that the signal would increase as the protein denatured, reach a peak, and then decline at the highest temperatures. Therefore, the third part of the graph would show a negative slope instead of forming a plateau. This decline in signal would change how the program interpreted the data, as it would not be able to fit a sigmoidal curve to calculate the melting temperature of the protein. In some cases where this happens, the decline in signal is due to the protein aggregating as they become denatured, thereby decreasing the total amount of surface area that interacts with the ANS in solution. In our case, the problem was most likely that there were temperature-dependent interactions that happened between the ANS and the compound, thereby allowing the ANS to give off a fluorescent signal when in contact with the compound rather than with the hydrophobic residues on the interior of the protein.

After this was noticed, the Up/Down Ramp setting was used for the remaining experiments. The Up/Down Ramp, instead of simply increasing the temperature over time, returns back to the lowest temperature set in the range before proceeding to the next step, in order to take fluorescent readings at a constant temperature. In some cases, this allows for the re-folding of the protein before each increase in temperature, thus preventing the aggregation that can be observed in the Continuous Ramp setting, and also preventing some of the interactions between the compound and ANS which influenced ANS's signal. While the Continuous Ramp gave us accurate and replicable results, the Up/Down Ramp allows us to more accurately fit a curve onto the data and obtain a consistent melting temperature for the protein.

The stabilization of the protein by the compound was quantified by calculating the difference in melting temperature between the protein-compound complex and the native protein. A positive change in melting temperature (ΔT_m) would indicate a stabilizing interaction, while a small or nonexistant change in temperature suggests little to no interaction between the compound and the protein. Negative ΔT_m values are interpreted as destabilizing interactions, meaning that the compound somehow influences the protein in a way that facilitates denaturation.

Phosphorylation Assay

GRKs are kinases, meaning that they phosphorylate proteins using ATP as a substrate. The phosphorylation assay uses four primary ingredients: the kinase (GRK2 or GRK5), a protein substrate (bovine rhodopsin [bROS] or tubulin), magnesium, and ATP. The magnesium in this situation acts as a cofactor for the reaction, as it binds to the enzyme with ATP. The ATP provides the phosphate group that will be transferred on the substrate. The use of radioactive

phosphorus allows us to track the movement of the phosphate group from the ATP to the substrate (Figure 7A). A large amount of radioactive material in the protein after the assay indicates a greater amount of catalytic activity. By comparing the levels of catalytic activity, we will be able to tell the amount of inhibition mediated by small molecules as they interact with the kinase as well as their potency.

Two different protein substrates were used for these reactions. Tubulin is a soluble protein that is easily phosphorylated by GRKs, although with less efficiency than GPCRs. The other substrate, bovine rhodopsin (bROS), is a readily available light-activated GPCR that serves as a substrate for all GRKs. Rhodopsin is expressed in the retina and enables low-light vision. Rhodopsin contains a chromophore, 11-cis-retinal, that undergoes an irreversible isomerization upon absorption of a photon of light. Therefore, this protein must be processed in the dark, and can only be exposed to light during the reaction (after ATP has been added). Despite their differences, each of these substrates is able to accurately measure the activity of the compound-bound protein, and both are used to add variability to the different experiments performed.

In these projects, phosphorylation assays were used to measure the potency of the compounds. Dose response curves were fitted for each of the compounds, allowing us to find IC_{50} values, quantifying the strength of the inhibitor in relation to catalytic activity. A series of dilutions were made for each of the compounds and plated in duplicate. Stock solutions for the GRK and the substrate were also made at this time using 20 mM HEPES pH 7.0, 2 mM $MgCl_2$. For the kinase, the stock concentration was 200 nM, with a final reaction concentration of 50 nM. The substrate was at a concentration of 2 μM, for a final reaction concentration of 500 nM. The compounds were then incubated with both the kinase and the substrate for ~30 min to allow for binding of the inhibitor.

An ATP stock solution was made up at 20 μM with an approximately 20 μCi of radioactive ATP added for every 150 μL of stock made. This ATP stock solution was added and allowed to react for a set amount of time before the reaction was quenched with SDS loading dye. Various tests were done to optimize the reaction time that allowed the kinase to act on the protein while still demonstrating the amount of inhibition provided by the compound. We wanted to keep the reaction in the "first-order", or linear, part of the overall reaction, which is where the velocity of the reaction is essentially constant. This optimization would result in a better fitting curve for the dose response and thereby allow for more accurate calculation of the IC_{50} values for each sample.

Each of the samples was run on a 12% SDS-PAGE gel at 225V for 45 min. The resulting gel was dried with a marker of 1:1000 diluted ATP stock. The marker would later be used to calculate the amount of radioactivity in each sample. The gels were then developed on a Storage Phosphor Screen (GE Healthcare), which would later be scanned using a Typhoon 9410 scanner (Amersham) for quantification of the radioactivity in each of the samples. Data was analyzed using ImageQuantTL software (GE Healthcare). From the quantified radioactivity levels for each sample, dose response curves could be formed to determine the IC_{50} values in relation to catalytic kinase activity (Figure 7B).

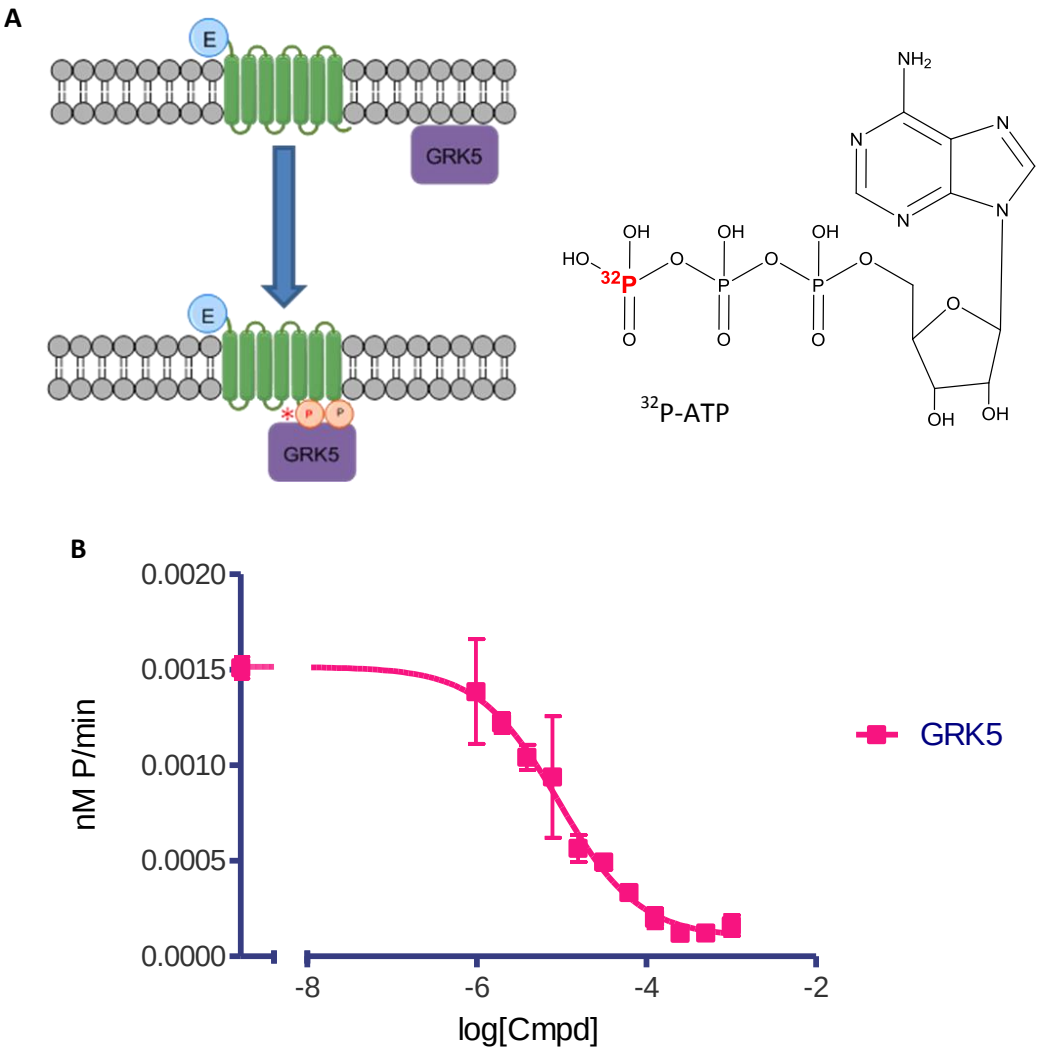


Figure 7. Phosphorylation assay is used to measure potency of each compound
(A) In phosphorylation assays, the activity of the kinase is measured using radioactive ^{32}P-ATP, whose terminal radioactive phosphate is incorporated into the rhodopsin or tubulin substrate. (B) The calculated dose response curve is generated as a measure of activity based upon different concentrations of inhibitor against the enzyme and permits the determination of IC_{50} values.

Results

The search for a specific inhibitor of GRK2 started from screening small molecule compound libraries, and grew more selective with each elimination. This was done with the ultimate goal of selecting a reasonable number of compounds for selectivity testing. Using a

range of different assays, potency, selectivity, and competitive binding were analyzed for a variety of compounds. We later progressed to a parallel project involving GRK5, using many similar techniques to screen for selective small molecule inhibitors, with our primary screening process being the major difference between the two. While GRK2 was screened using a competition based assay, GRK5 was screened based on protein-compound stabilizing interactions.

Flow Cytometry Assay Development

One of the main assays used in screening for GRK2 inhibitors was FCPIA. Before testing began on compounds, the FCPIA was tested using some of the previously studied binding agents and inhibitors of GRK2. These included ATP, unlabeled aptamer (C13.28), sangivamycin[40], and the Takeda compound 103A (Figure 8).

It was found that the most potent competitors of C13.28-FAM binding were unlabeled C13.28 (pIC_{50} = 9.3) and 103A (pIC_{50} = 8.6), which were able to completely reduce the fluorescence signal to the positive control levels. Lower affinity compounds ATP (K_m=28 μM) and sangivamycin (IC_{50} =70 μM) were only able to decrease fluorescence by 40-70% over the concentrations evaluated. Although these two were able to decrease affinity between the aptamer and GRK2, they did not fully compete, indicating allosteric modulation of aptamer binding. Their competition with C13.28-FAM resulted in pIC_{50} values of 4.3 and 5.0 respectively.

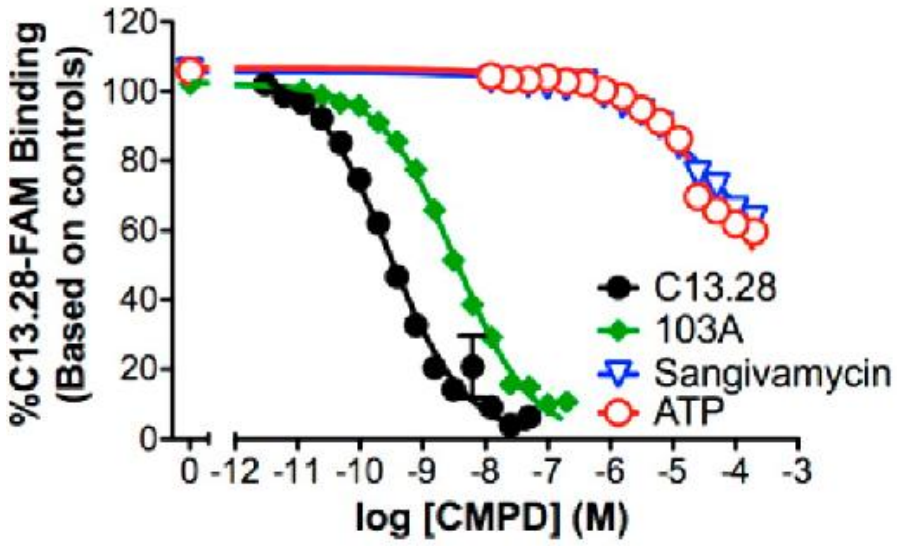


Figure 8. Tests of known inhibitors of GRK2 in the aptamer displacement assay
The inhibitors sangivamycin and Takeda compound 103A were used in preliminary testing, along with ATP and unlabeled C13.28 aptamer. [49]

Primary Screen of GRK2 against the Center for Chemical Genomics (CCG) Compound Library

We began the search for inhibitors by conducting FCPIAs on GRK2 against a collection of ~40,000 compounds. These compounds were part of a larger 100,000 compound library purchased from ChemDiv and housed at the CCG at the University of Michigan's Life Sciences Institute. Out of the ~40,000 compounds that were tested in this preliminary screening, we identified 6017 compounds that blocked the binding of the RNA aptamer inhibitor C13.28-FAM to the active site of GRK2 with a standard deviation > 3 by plate (Figure 9).

After conducting the counterscreen, we found that a large number of the compounds interfered directly with the fluorescence signaling by directly interacting with the C13.28-FAM. For this round of selection, new parameters were placed in order to narrow down a more potent and specific list of compounds for a second round of screening. Using a cutoff of standard deviations greater than 3 and greater than 20% activity based on the controls of each plate, we selected 1811 compound hits for continued testing. These compounds were then put through a

FCPIA confirmation screen, in which they were plated and tested in triplicate at a concentration of 100 μM. 412 of the remaining compounds were found to retain greater than 20% activity based on three independent experiments.

In a collaboration with the University of New Mexico's Center for Molecular Discovery, a separate set of 1,200 compounds from the university's Prestwick Chemical Library was also screened in the same manner as the 40,000 ChemDiv Library. Similar parameters were put into place for identification of hits from this library.

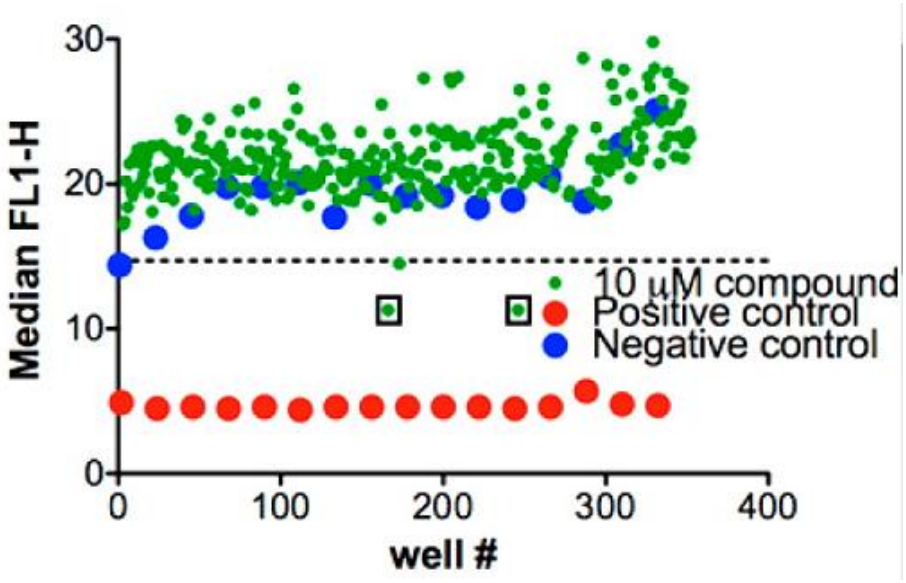


Figure 9. Primary screening of GRK2 using FCPIA
In a single-point FCPIA, competitive binding is measured in relation to the positive and negative controls (10 μM C13.28 and DMSO respectively). In the primary screen, the definition of a "hit" involved looking at the standard deviation of the fluorescence reading from the negative control. Positive hits are shown here in boxes.[49]

FCPIA Dose Response Titrations of GRK2

Dose response curves were performed in order to test the potency of the 412 selected compounds from the CCG on GRK2. Each of the selected compounds was serially diluted in duplicate down the plate with a highest concentration of 50 μM and a lowest concentration of 0.78 μM and levels of the fluorescence were measured at each concentration. From the FCPIA data collected from these plates, we were able to build dose response curves that told us how well each compound bound relative to the other compounds. This was measured and quantified using a half-maximal inhibition concentration value (IC_{50}) which is the concentration required for 50% inhibition based on plate controls. Within this set of 412 compounds, the cutoff was set at a pIC_{50} ($-\log IC_{50}$ value) of 4 or greater. This narrowed the field of samples down to 237 compounds.

Dose response curves were also generated a different set of 20 compounds from a screen of the ChemDiv 100,000 compound library (Figure 10). With a highest concentration of 100 μM and a lowest concentration of 0.78 μM, these sets were done in duplicate. The control aptamer screen was also performed on this set of compounds in dose titration format, in order to clearly assess the results gained from the GRK2 assay. Some of the problems that arose from this particular set of compounds involved solubility issues. For this particular experiment, the compounds had to be soluble in 200 μM stock solutions. In order to prevent complete precipitation, a few of the compounds had to be solubilized with as much as 33% DMSO. This posed a problem with regards to the DMSO affecting the state of the protein, and this caveat had to be taken into account when assessing the results.

From this set of 20 compounds, we found that there were several compounds with low IC_{50} values that were to be further examined. From those that seemed to have lower concentration IC_{50} values, the control screen against the aptamer demonstrated that these hits

were compounds that interfered directly with the aptamer or quenched the fluorescence signal, and did not interact with the protein. In later experiments where three of the 20 compounds were re-examined in dose response with a highest concentration of 100 µM, it was found that their IC_{50} values only came to 50 µM or higher, which is not low enough to be considered for pharmacological testing. These compounds were thus determined as not worthy of further pursuit, and the project shifted its focus back onto the hits from the 40,000 compound screen.

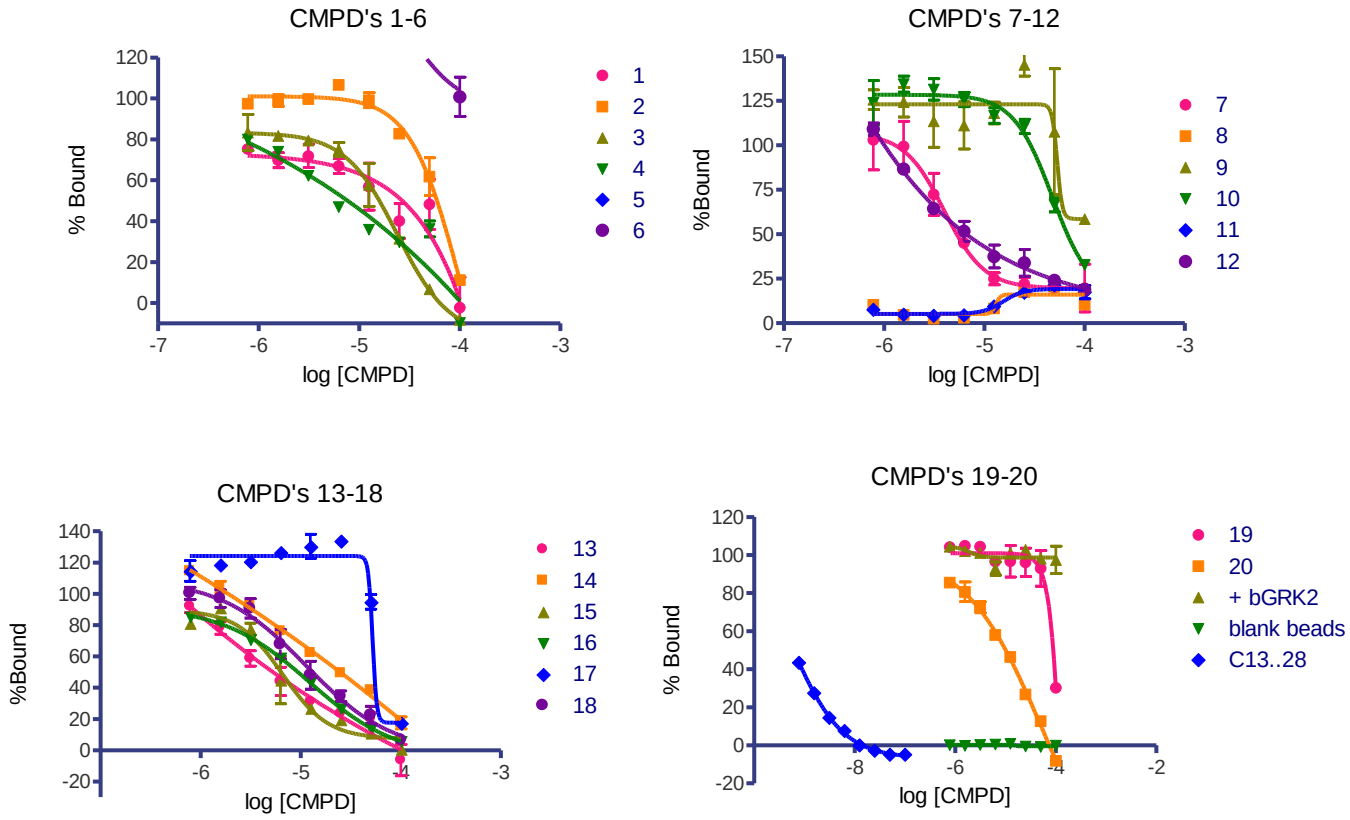


Figure 10. Dose response curves from 20 compounds from a ChemDiv screen
Subsequent experiments revealed that the hits from this set of compounds also interfered directly with the C13.28 aptamer. None of these compounds were tested further.

GRK2 Thermofluor Screening

Out of the 40,000 compounds originally screened, we found 237 compounds that were capable of displacing the RNA aptamer from the active site of GRK2 in dose dependent manner. These compounds were then selected for additional testing with a Thermofluor assay. The compounds were each tested in triplicate over a temperature range of 25˚C-70˚C.

Compounds were selected for advancement if they had a Hill slope between -2 and 2, IC_{50} values ≤30 μM, and median ΔT_m values >0.16˚C. From this list of 237 compounds, 37 were selected. Out of these, only 32 compounds were purchased due to their market availability.

FCPIA Dose Response Titrations of Selected Compounds Against GRK2

As a result of the collaboration with the University of New Mexico's Center for Molecular Discovery, two compounds were found in the Prestwick Collection that were able of efficiently displacing the RNA aptamer. These compounds (P-851 and P-835) were confirmed with dose response titrations using the FCPIA with a highest concentration of 100 μM. A control dose response was also done using the b-C13.28-FAM and the compounds did not demonstrate any significant interaction or interference with the aptamer. The controls used for this plate were the same as previously used positive and negative controls of no GRK2 and no compound interference, as well as a separate set of controls composed of the known GRK2 inhibitors balanol and Takeda compound 103A. These inhibitors were used in order to compare the shape and the magnitude of the responses of the test compounds.

These dose response curves were performed in triplicate and were used to narrow down the field of compounds. Any compounds that consistently demonstrated dose dependent effects and were definitively proven to not interfere with the aptamer were selected for additional

analysis. From this selection, the number went from 34 compounds (2 identified at UNM and 32 we purchased as described above) to a set of 15, which included the 2 compounds found by the University of New Mexico. One of these compounds, P-851, shown in Figure 11, was among the strongest compounds identified with a measured pIC_{50} value of ~4.5 ± 0.2.

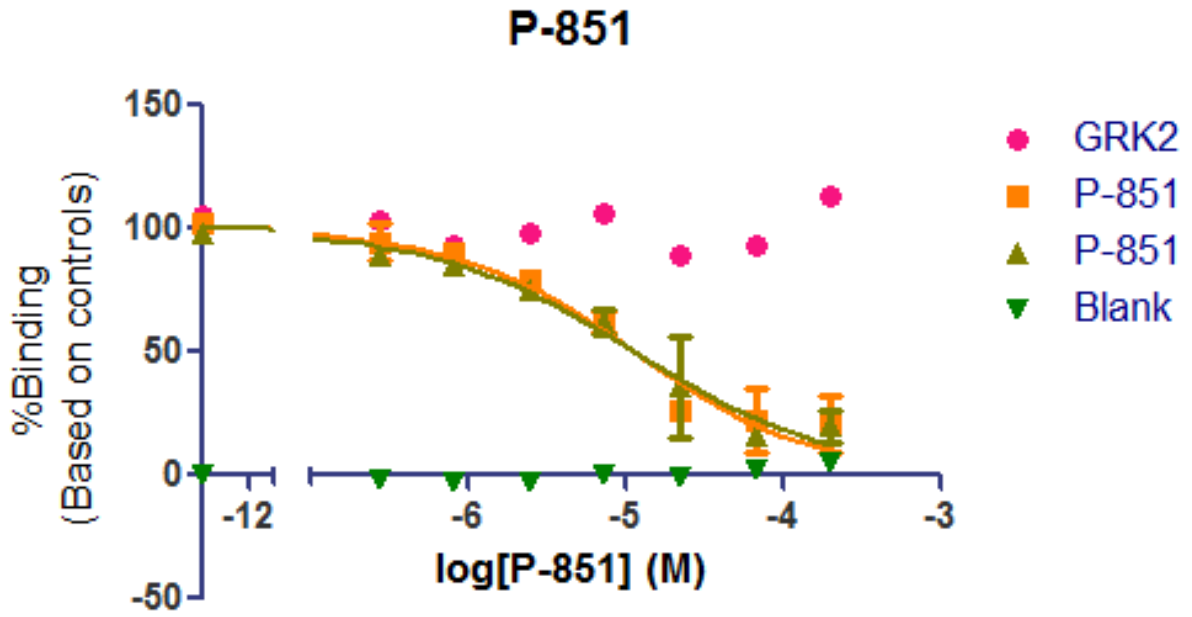


Figure 11. Paroxetine FCPIA dose response
FCPIA Dose Response data for compound P-851 (paroxetine) consistently showed a pIC_{50} value of 4.5 ± 0.2. This compound was followed up on with thermofluor testing.

Thermofluor Dose Dependence

By comparing the changes in T_m at a few concentrations, we can get a relative idea of the potency of each compound. Unlike FCPIA, dose response data in thermofluor is not considered as reliable due to the fact that changes in T_m based on compound concentration happen in too narrow of a concentration range. As a result, the dose response curve for thermofluor is sometimes considered too steep to be a reliable source. For our purposes, a few concentrations were tested to look at the dose dependence of protein-compound stability.

The 15 remaining compounds were tested at varying dosages to check for potency in thermofluor. The concentrations used were 200 µM, 100 µM, and 50 µM (Figure 12). Within this range of concentrations, variation between the T_m values of each compound was generally not significant. In thermofluor screening, the values that are considered "significant" are dependent on the protein and the substrates being tested. For GRK2, many hits for thermofluor testing have ΔT_m values <1˚C, with 1˚C considered as a significant shift for this particular assay. In striking contrast to the other compounds, P-851 consistently shifted the melting temperature of GRK2 by ~7.8˚C for each concentration. For comparison, ATP, the native substrate for GRK2, gives a T_m shift of 1.5-5.6˚C at these concentrations, with a ΔT_m of ~4.9˚C at 200µM ATP-Mg^{2+}. This suggests that P-851 has high affinity for GRK2.

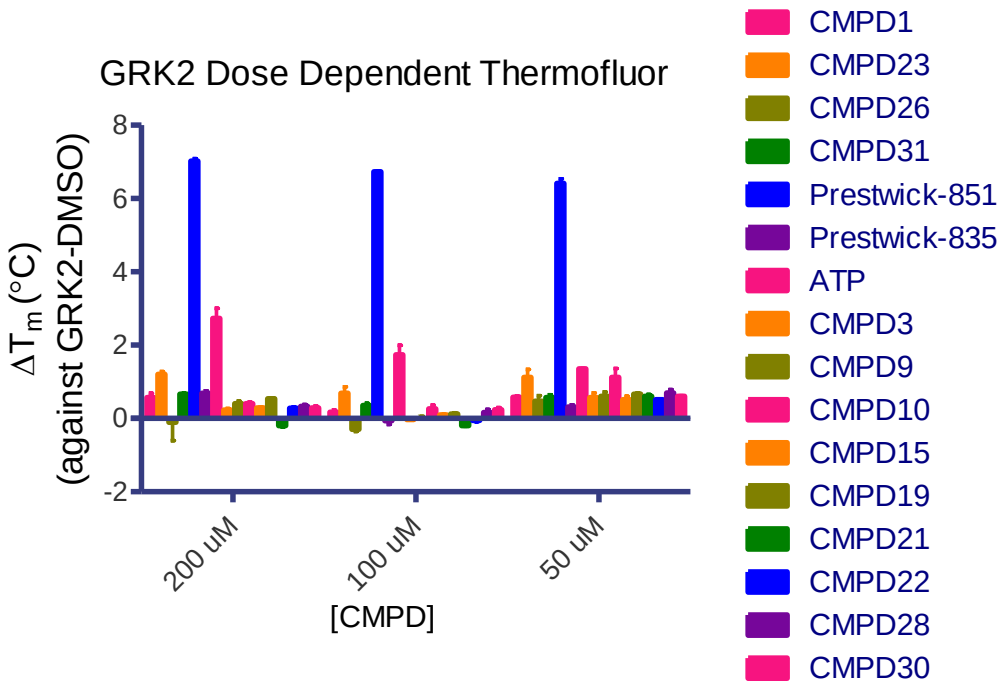


Figure 12. Dose dependent thermofluor
Thermofluor data measuring dose dependence for the selected 15 compounds at concentrations of 50µM, 100µM and 200µM was measured and compared for interaction strength.

Thermofluor Selectivity

The dose dependent thermofluor data yielded 6 compounds that were considered worth pursuing in additional GRK selectivity experiments. 5 of the compounds increased the stability of the structure and gave positive ΔT_m values, while 1 compound was categorized as a destabilizing molecule, showing a negative ΔT_m value. Destabilizing compounds, while not generally applicable as inhibitors, can be used to understand interactions between the protein and the compound. By understanding these interactions and those of the stabilizing compounds as well, we could potentially use these structures as the foundation for drug design research. These 6 compounds along with ATP were screened at a concentration of 200 μM against GRK1 and GRK5, representative members of the other GRK subfamilies (Figure 13).

4 of the compounds seemed to form more selective interactions, having a more stabilizing effect on GRK2 than on the other GRK subtypes. These included Compound 1 ($\Delta T_m = 0.40^{\circ}C$), Compound 23 ($\Delta T_m = 0.99^{\circ}C$), Compound P-851 ($\Delta T_m = 7.8^{\circ}C$), and Compound P-835 ($\Delta T_m = 0.73^{\circ}C$). This was in contrast to the other GRK subtypes, with GRK1 having melting temperature shifts of -0.09˚C (Compound 1), -0.66˚C (Compound 23), 0.95˚C (Compound 851), and -0.15˚C (Compound P-835), and GRK5 melting temperature shifts with -1.55˚C (Compound 1), -0.37˚C (Compound 23), -0.35˚C (Compound P-851), and -0.01˚C (Compound P-835). The single destabilizing compound was not found to be selective and was dismissed from further investigation.

From this set of experiments, P-851 continued to stand out as a particularly strong inhibitor candidate. From the thermofluor selectivity data, P-851 demonstrated selective stabilization of GRK2 in comparison to the other GRK subgroups, with a $\Delta T_m < 1^{\circ}C$ for both

GRK1 and GRK5. P-851 also managed to induce melting temperature shifts which were larger than those made by ATP binding to GRK2.

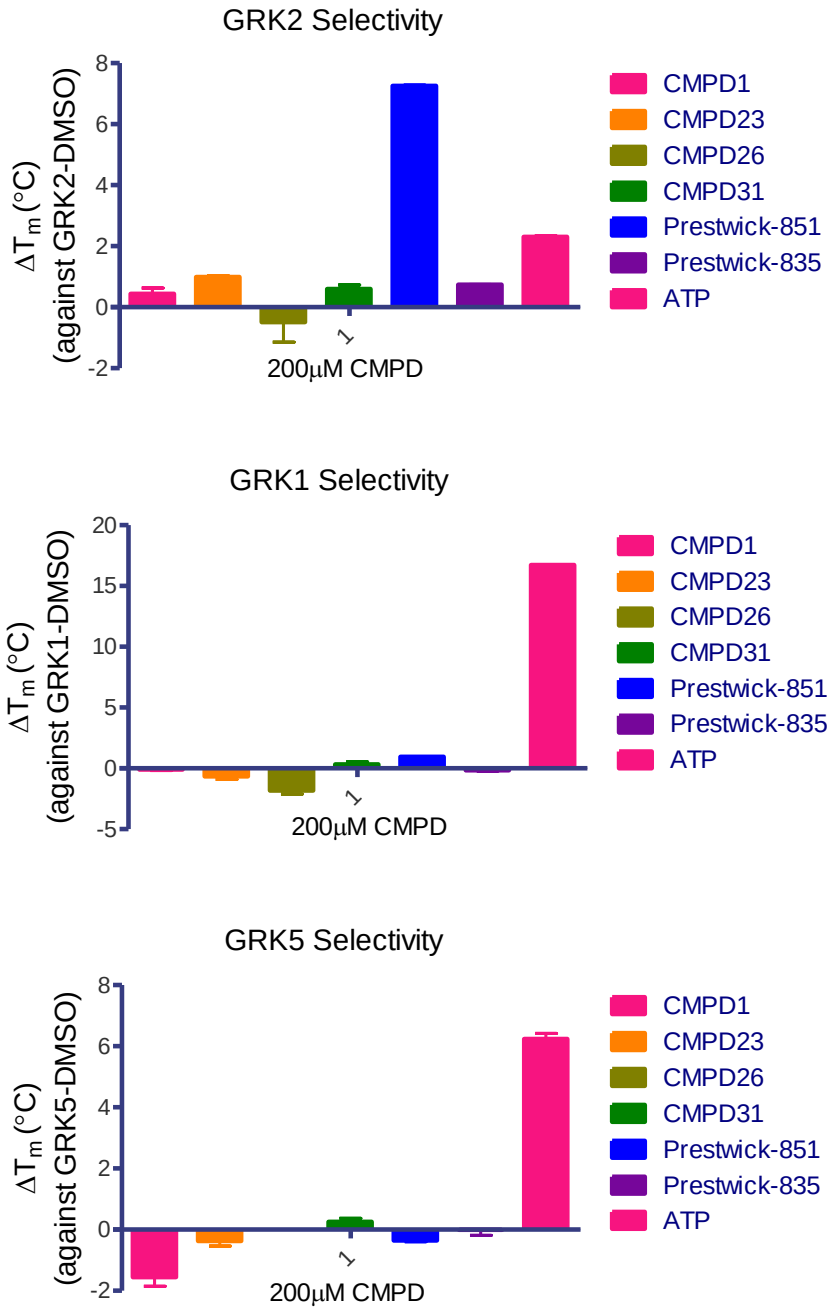


Figure 13. Thermofluor GRK selectivity data of 6 compounds identified in the FCPIA analysis
The 6 compounds were tested in thermofluor for stabilization data against representative members of all 3 GRK subfamilies. 4 out of the 6 chosen compounds were found to be selective for GRK2 against GRK1 and GRK5.

Compound P-851 stood out in particular, with a ΔT_m value of 7.8˚C for GRK2, while having a ΔT_m <1˚C for both of the other GRK subfamilies.

Compound P-851 (Paroxetine) Structure Activity Relationships

P-851 (henceforth called paroxetine) consistently demonstrated in FCPIA a pIC_{50} value of 4.5 ± 0.2 (Figure 11) and a ΔT_m of 7.8˚C in Thermofluor analysis (Figure 12). With follow-up analysis, a crystal structure of the protein-compound complex was obtained by Dr. David Thal in the lab, which demonstrated selective binding of paroxetine to GRK2 in a novel conformation[49].

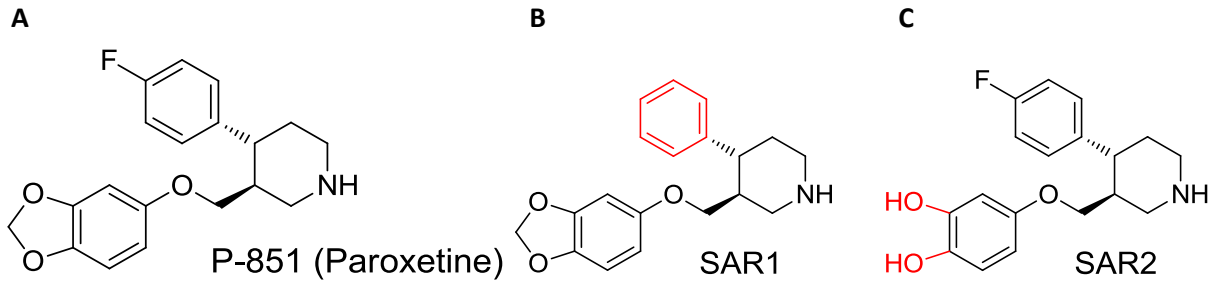
Following the elucidation of the structure, we began analyzing structural activity relationships (SARs), structural analogs of paroxetine, in an attempt to determine the important binding sites of the protein-compound complex. SAR1 is a defluorinated form of paroxetine, which was predicted to reduce multiple hydrophobic interactions between the compound and GRK2 (Figure 14B), while SAR2 is a desmethylene paroxetine which was predicted to reduce favorable van der Waals interactions within the active site of GRK2 (Figure 14C).

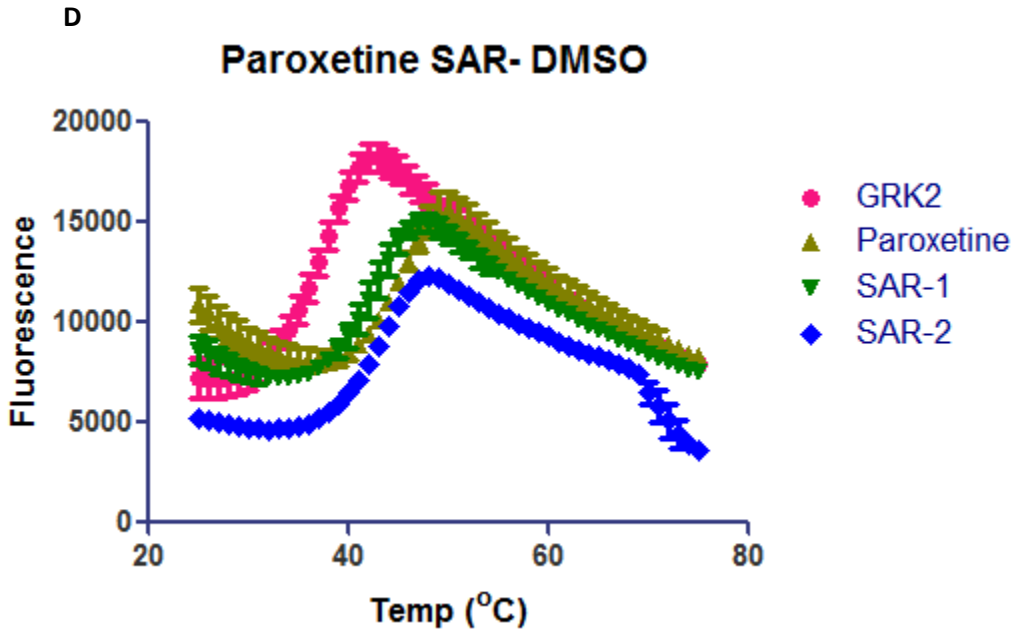
For primary analysis, thermofluor assays were run, comparing the binding and stabilizing strength of the SAR compounds against the original hit. Both SAR compounds showed decreased thermostabilization as well as decreased inhibition of GRK2 activity in relation to the original compound due to fewer intermolecular interactions with the binding site of the protein.

In thermofluor, in comparison to the 7.8˚C increase in T_m given by the paroxetine-GRK2 interaction, the SAR interactions were proven to be weaker, as predicted. This was indicated by the decrease in ΔT_m values for the analogous compounds, with SAR1 found to be a ΔT_m of 4.91˚C, while SAR2 provided a ΔT_m value of 5.54˚C (Figure 14D and 14E). Although these are still relatively large shifts in melting temperature against GRK2, they are still decreased in

relation to the original paroxetine structure which had more optimized interactions between the compound and the protein.

Follow-up activity analysis of SAR compounds concluded my direct involvement with this particular project, which in turn was followed up with in vivo studies performed by a collaborator at Temple University. It was proven that inhibition of overexpressed GRK2 in vivo using paroxetine helps to increase myocardial contractility[49].





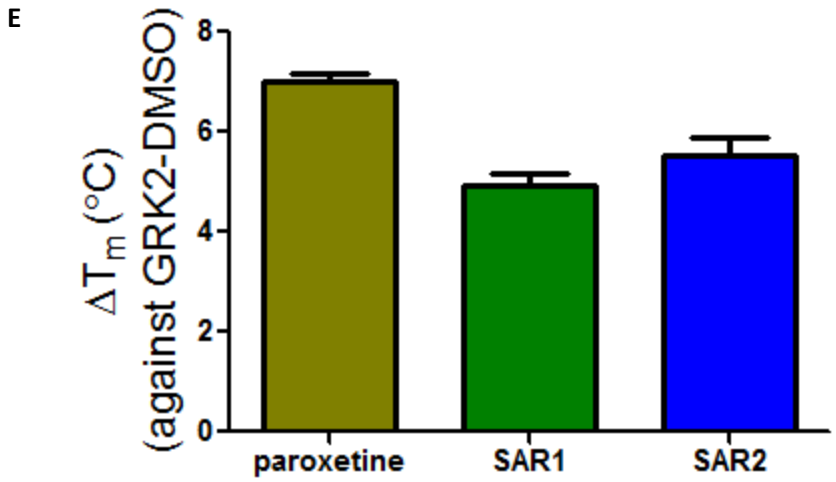


Figure 14. Paroxetine structure activity relationships
(A) Paroxetine stood out as a particularly potent compound against GRK2. Structure activity relationships (SARs) were studied to determine the nature of the interaction between the compound and GRK2. (B) SAR1 is a defluorinated form of paroxetine, while (C) SAR2 is a desmethylene paroxetine. (D and E) As predicted, interactions between the SAR compounds and GRK2 were weaker in strength than those of the original paroxetine against GRK2. The difference in affinity can be seen by a decrease in melting temperature of the protein-compound complex.

GRK5 Thermofluor Screening

After the analysis of GRK2 inhibition with P-851, we started work on a parallel and related project regarding another member of the GRK family, GRK5. GRK5 represents a different subfamily of the GRKs, potentially suggesting unique binding mechanism. GRK5 has exhibited regulatory functions in the heart and lungs similar to those of GRK2, suggesting that inhibition of GRK5 would result in therapeutic protection against heart failure. As of yet, no small molecule inhibitors of this protein have been identified. GRK5 also lacks a known structure, and finding a molecule which binds to the active site could potentially facilitate a structure determination.

The search for a small molecule inhibitor began with the screening of a 16,000 compound library from the CCG against GRK5 protein using the thermofluor assay. Preliminary findings from previous studies done within the lab have shown that GRK5 has shown promising

thermostabilization data against both a negative control of DMSO and a positive control of ATP, which is known to bind at the active site[49] (Figure 15A). Although analysis of certain compounds for selectivity has been targeted for GRK2 binding, it has been shown that counter-screening against GRK5 is capable of identifying specific binding against different GRKs using this assay. Thus, it is as an appropriate test for potential inhibitors of the kinase activity.

Primary screening on the library began by performing a four-fold multiplex test in thermofluor, which involved incubating four compounds per sample of GRK5. Controls for this experiment were again DMSO as a negative control and 5 mM ATP as a positive control. 5mM ATP with GRK5 gives a ΔT_m value of 15.5˚C, with GRK5's native melting temperature with a DMSO control being 25.9˚C. Some concerns were raised regarding the presence of both a stabilizing compound and a destabilizing compound being screened in one sample. Although interference between the compounds interacting with each other or conflicting with multiple interactions on the protein is possible, these concerns were seen as negligible, as the likelihood of a strongly stabilizing and a strongly destabilizing compound coming together at random was unlikely.

From the original 16,000 compounds, 60 samples (240 individual compounds) were initially identified as potential thermo-modulating compounds. These compounds were selected based a criteria of $\Delta T_m > 1.7$˚C or $\Delta T_m < -4.9$˚C, 2.5 standard deviations away from the native melting temperature of the protein. Both stabilizing and destabilizing compounds were selected for continued analysis. The destabilizing compounds of GRK5 were chosen for the benefit of analyzing inferring interactions between the compound and the protein.

As an example, one of the hits that we obtained was found to contain a well known kinase inhibitor, staurosporine, which gave a multiplex melting temperature shift of 4.4˚C

(Figure 15B). This was taken as a proof of concept with regards to screening for inhibitors against GRK5 using thermofluor stabilization.

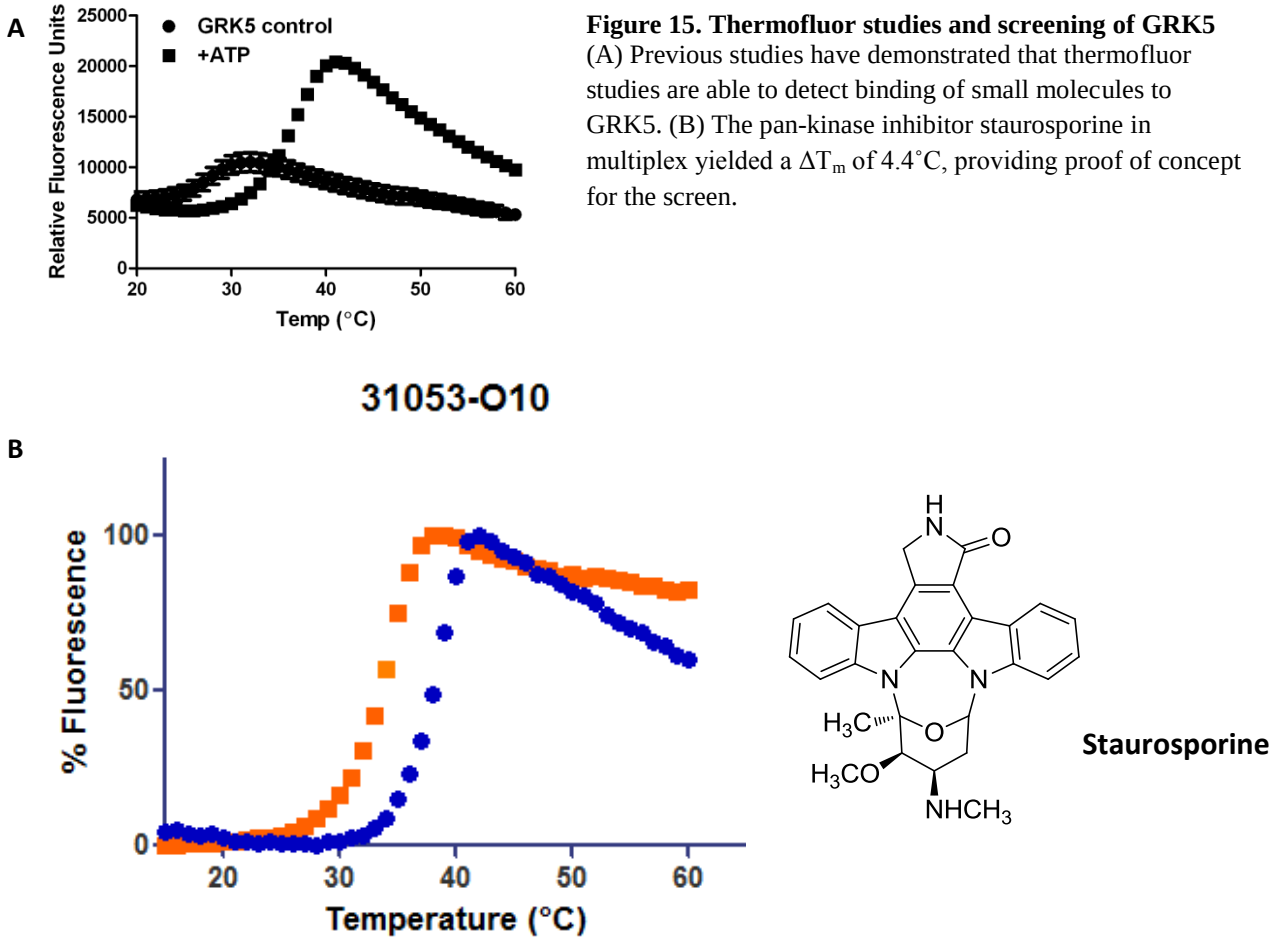


Figure 15. Thermofluor studies and screening of GRK5 (A) Previous studies have demonstrated that thermofluor studies are able to detect binding of small molecules to GRK5. (B) The pan-kinase inhibitor staurosporine in multiplex yielded a ΔT_m of 4.4˚C, providing proof of concept for the screen.

Confirmation Thermofluor for GRK5

These hits were deconvoluted into their individual compounds and tested in a confirmation thermofluor assay, with each individual compound tested in quadruplicate. The concentration of these compounds was 10 μM, slightly less concentrated than the GRK2 compounds that were retested based on expected potency of the compounds. Each quadruplicate was analyzed as a set, with hits defined as needing significant shifts in 2 or more of the 4

samples. Using parameters of 2.5 standard deviations with $\Delta T_m > 12\%$ or $\Delta T_m < -35\%$ based on the controls of DMSO and ATP, we narrowed it down to 20 compounds for follow up in a final thermofluor trial (Figure 16). These hits were composed of 17 positive shifts in melting temperature and 3 destabilizing hits.

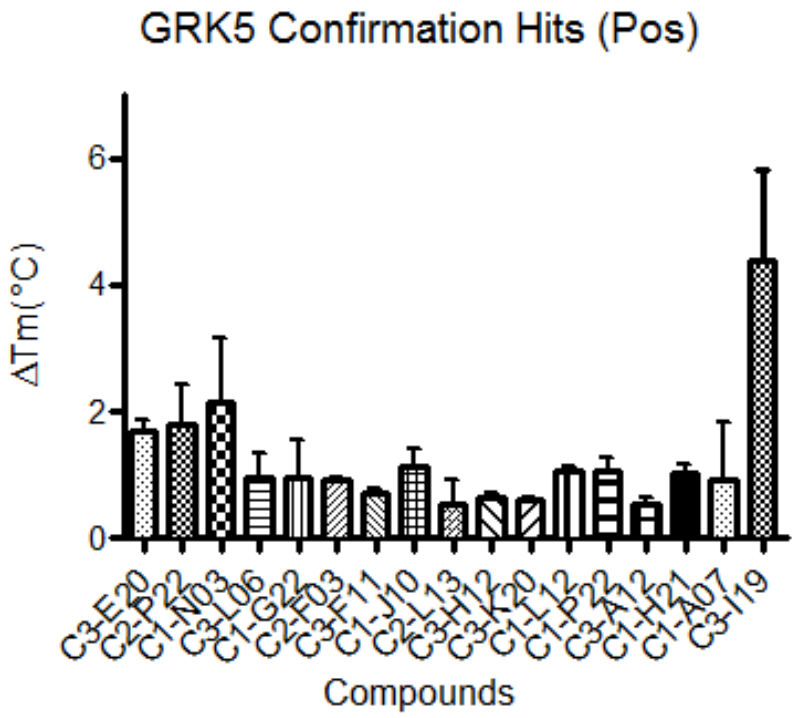


Figure 16. GRK5 confirmation hits
Based on the deconvolution of hits found from the multiplex screen, 20 compounds were confirmed as hits for the assay. Out of these 20, 17 of them were stabilizing hits that gave positive ΔT_m values, qualifying them as potential inhibitors. The other three hits were destabilizing compounds which were investigated for protein-compound complex interactions.

Dose dependence testing in thermofluor was the final step in observing the stabilizing strength of the compounds within a range of concentrations. Similar to what was done with the GRK2 compounds, the 20 compounds were tested in a variety of concentrations ranging from 1

μM to 120 μM. This once again allowed us to look at relative potency in a broad context without determining an exact IC_{50} value.

From these final results, 4 compounds were found that were selected as possible inhibitors for GRK5. These results included byssochlamic acid (Figure 17A), amlexanox (Figure 17B), Box5 (Figure 17C), and CCG-5274-0479 (Figure 17D).

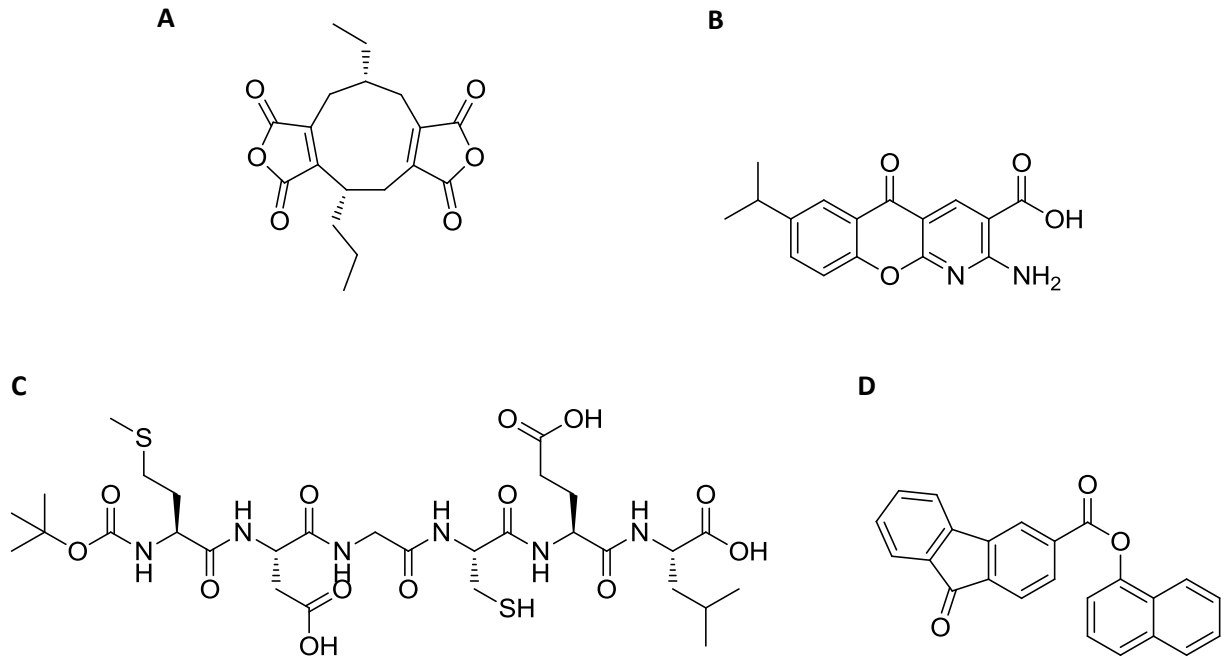


Figure 17. GRK5 dose dependence
Dose dependence testing resulted in 4 selected compounds for further analysis in phosphorylation assays. These compounds included (A) byssochlamic acid, (B) amlexanox, (C) Box5, and (D) CCG-5274-0479. Out of these compounds, amlexanox was chosen as the one compound that merited the most attention in further analysis.

Phosphorylation Screening of GRK5 with Amlexanox

Ultimately only amlexanox was chosen for further analysis with GRK5 inhibition activity. This was based on a variety of factors mostly involving the compatibility of the compounds with biological systems. Byssochlamic acid, with a large nonane ring structure and

reactive acid anhydride groups was determined to most likely be toxic in a biological system. Box5, while not toxic, has a modified peptide structure that makes it more difficult to deliver into a cell. This lack of bioavailability eliminated box5 from current avenues of investigation.

Phosphorylation assays at various concentrations of amlexanox were done to assess stabilization and relative potency of the compound. Amlexanox concentrations were tested within a range of 125 nM to 1 mM using two-fold dilutions. Through analysis of phosphorylation signal over the various concentrations, it was found that amlexanox bound GRK5 with an IC_{50} of 10 μM (Figure 18).

This gave us enough reason to look into the selectivity of the compound against the other GRK families. Using the same techniques with amlexanox against GRK1 and GRK2, we found that the dose response curves did not converge for these GRKs, indicating that amlexanox does not bind or inhibit other GRK subfamilies. This demonstrated that amlexanox forms a unique and stabilizing interaction with GRK5 in relation to the other members of the GRK family. It also shows that GRK5 is the only GRK catalytically inhibited by amlexanox, indicating remarkable specificity.

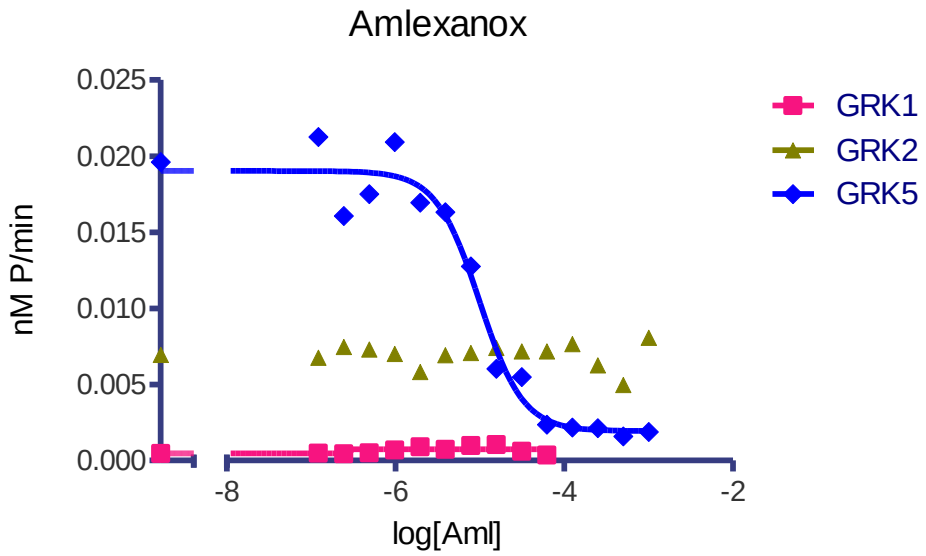


Figure 18. GRK5 phosphorylation with amlexanox
Amlexanox was tested for inhibition of catalytic activity using phosphorylation assays. Compound potency was tested for over a range of concentrations (125 nM-1 mM). GRK5 was shown to have an IC_{50} value of 10 μM with Amlexanox. Selectivity was tested by analyzing inhibition of other members of the GRK family. GRK1 and GRK2, representatives of the other subfamilies, were not inhibited by Amlexanox.

Discussion

GRK2 and GRK5 have both been studied extensively for their regulation of cardiac contractility. Kinase inhibitors in general have received a lot of attention by the pharmacology community, composing over 25% of all drugs on the market[52]. Most studies aim to find inhibitors which directly interact with the active site of the enzyme. This approach is successful in finding inhibitors for the kinase activity, but they tend to be nonspecific small molecule inhibitors which compete directly with ATP, and hence potentially with many other enzymes that bind ATP.

At the same time, most of the medications which are directed towards preventing or treating heart disease are β-blockers, compounds aimed at binding and directly inhibiting the β-adrenergic receptors. While these have proven effective in helping to reduce the rates of

morbidity and mortality in patients suffering from congestive heart failure[53], there are certain detrimental effects that can result from taking these drugs[54].

Using this novel RNA aptamer, C13, our lab was able to target a non-traditional part of the sympathetic signaling pathway. Because of the fact that C13 binds and inhibits GRK2 selectively by inducing a novel inactive conformation of the protein, screening compounds in competition with the aptamer implies a much more selective set of hits. This means that in conjunction to avoiding the β-adrenergic receptor target, the aptamer ultimately allows us to eliminate from the beginning a subset of the non-specific inhibitors which generally associate with the more conserved regions of the kinase, the active site associated with ATP binding.

Through this method of screening, we were able to find one particular compound which demonstrates the ability to directly affect the catalytic activity of GRK2 through direct binding and stabilization of a novel conformation. Paroxetine has been shown to compete off the aptamer with high potency and thermostabilize GRK2 to a greater extent than its native substrate, ATP. In a crystallographic study, paroxetine was observed to induce a novel conformation of the protein while binding in the ATP binding site[49]. This discovery of a novel conformation which drastically stabilizes and inactivates could potentially act as a foundation for future drug design with regards to GRK2. Understanding the intermolecular interactions formed by this compound will hopefully lead to their optimization in future synthetic studies, thereby providing the gateway to a more selective inhibitor of the sympathetic nervous system.

Paroxetine, as an antidepressant drug, has been on the market since 1992. While it has been proven to have these effects on cardiac contractility, there has been no reported correlation between paroxetine use and decreased heart failure. In canine models, it has been estimated that the blood plasma levels of paroxetine is around 125 nM, which is several factors below the 30

μM levels estimated for effectiveness against GRK2. However, these levels are not uniform throughout the body, with paroxetine concentrating at certain target areas such as the CNS[55]. With the discovery of its affinity for GRK2, the effects of paroxetine might now take on a new significance. Future directions on this project might include a surveyed investigation of the correlation between heart failure rates of patients on paroxetine and those on other β-blocker medications.

Exploring the selectivity with regards to binding specificity against other GRK proteins also helps us to understand the distinctive structural elements which define the different GRKs. As of now, no crystal structure for GRK5 has been generated. This gives the search for an interactive compound a slightly different purpose with regards to protein-compound interactions. One of the ideas behind looking into molecules which bind GRK5 is the possibility of co-crystallizing the two and investigating a more stabilized conformation of the structure. By elucidating a unique conformation of the protein, we have the potential of generating a model to work off of when looking into the native structure of the protein itself and to design new drugs.

References

1. "The Nobel Prize in Chemistry 2012". Nobelprize.org. 9 Nov 2012. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2012/
2. Wu G (2010). "Assays with GPCRs". *Assay Development: Fundamentals and Practices*. New York: Wiley. ISBN 0-470-19115-5.
3. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ (June 1990). "β-Arrestin: a protein that regulates β-adrenergic receptor function". *Science* 248 (4962): 1547–1550.
4. Tan CM, Brady AE, Nickols HH, Wang Q, Limbird LE (2004). "Membrane trafficking of G protein-coupled receptors". *Annu. Rev. Pharmacol. Toxicol.* 44: 559–609.
5. Luttrell LM, Lefkowitz RJ (February 2002). "The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals". *J. Cell. Sci.* 115 (Pt 3): 455–65.
6. Liggett SB, Cresci S, Kelly RJ, Syed FM, Matkovich SJ, Hahn HS, Diwan A, *et al.* A GRK5 polymorphism that inhibits beta-adrenergic receptor signaling is protective in heart failure. Nat Med 2008, 14: 510-517
7. Tesmer, V.M., Kawano, T., Shankaranarayanan, A., Kozasa, T., Tesmer, J.J. Snapshot of activated G proteins at the membrane: The Gαq-GRK2-Gβγ complex. Science. 2005;310:1686–1690.
8. Ma, Lan, Jianxia Gao, and Xiaoqing Chen. "G Protein-Coupled Receptor Kinases." *The G Protein-coupled Receptors Handbook*. By Lakshmi A. Devi. Totowa, NJ: Humana, 2005.
9. Dorn GW II. GRK mythology: G-protein receptor kinases in cardiovascular disease. *J Mol Med.* 2009;87:455– 463.

10. Metaye T, Gibelin H, Perdrisot R, Kraimps JL. Pathophysiological roles of G-protein-coupled receptor kinases. *Cell Signal*. 2005;17:917–928.
11. Penela P, Murga C, Ribas C, Lafarga V, Mayor F Jr. The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol*. 2010;160:821– 832.
12. Kunapuli, P., Benovic, J.L. Cloning and expression of GRK5: A member of the G protein-coupled receptor kinase family. Proc. Natl. Acad. Sci. 1993;90:5588–5592.
13. Brodde OE. Beta-adrenoceptors in cardiac disease. *Pharmacol Ther*. 1993;60:405– 430.
14. Gauthier C, Tavernier G, Charpentier F, Langin D, Le Marec H. Functional beta3-adrenoceptor in the human heart. *J Clin Invest*. 1996;98: 556–562.
15. Bristow MR, Minobe WA, Raynolds MV, Port JD, Rasmussen R, Ray PE, Feldman AM. Reduced beta 1 receptor messenger RNA abundance in the failing human heart. *J Clin Invest*. 1993;92:2737–2745.
16. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N Engl J Med*. 1982;307:205–211.
17. Bristow MR, Hershberger RE, Port JD, Gilbert EM, Sandoval A, Rasmussen R, Cates AE, Feldman AM. Beta-adrenergic pathways in nonfailing and failing human ventricular myocardium. *Circulation*. 1990; 82:I12–I25.
18. Ungerer M, Bohm M, Elce JS, Erdmann E, Lohse MJ. Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation*. 1993;87:454–463.

19. Landmesser U, Drexler H. Update on inotropic therapy in the management of acute heart failure. *Curr Treat Options Cardiovasc Med*. 2007;9:443– 449.
20. Dzimiri N, Muiya P, Andres E, Al-Halees Z. Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases. *Eur J Pharmacol*. 2004;489:167–177.
21. Pleger ST, Boucher M, Most P, Koch WJ. Targeting myocardial beta-adrenergic receptor signaling and calcium cycling for heart failure gene therapy. *J Card Fail*. 2007;13:401–414.
22. Ungerer M, Kessebohm K, Kronsbein K, Lohse MJ, Richardt G. Activation of beta-adrenergic receptor kinase during myocardial ischemia. *Circ Res*. 1996;79:455– 460.
23. Iaccarino G, Keys JR, Rapacciuolo A, Shotwell KF, Lefkowitz RJ, Rockman HA, Koch WJ. Regulation of myocardial betaARK1 expression in catecholamine-induced cardiac hypertrophy in transgenic mice overexpressing alpha1B-adrenergic receptors. *J Am Coll Cardiol*. 2001;38:534 –540.
24. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. *Proc Natl Acad Sci U S A*. 2001;98:5809 –5814.
25. Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J Jr, Lefkowitz RJ, Koch WJ. Expression of a beta-adrenergic receptor kinase1 inhibitor prevents the development of myocardial failure in gene targeted mice. *Proc Natl Acad Sci U S A*. 1998;95:7000 – 7005.
26. Brinks H, Koch WJ. BetaARKct: a therapeutic approach for improved adrenergic signaling and function in heart disease. *J Cardiovasc Transl Res*. 2010;3:499 –506.

27. Petrofski JA, Koch WJ. The beta-adrenergic receptor kinase in heart failure. *J Mol Cell Cardiol*. 2003;35:1167–1174.
28. Anderson KM, Eckhart AD, Willette RN, Koch WJ. The myocardial beta-adrenergic system in spontaneously hypertensive heart failure (SHHF) rats. *Hypertension*. 1999;33:402– 407.
29. Ping P, Anzai T, Gao M, Hammond HK. Adenylyl cyclase and G protein receptor kinase expression during development of heart failure. *Am J Physiol*. 1997;273:H707–H717.
30. Harris CA, Chuang TT, Scorer CA. Expression of GRK2 is increased in the left ventricles of cardiomyopathic hamsters. *Basic Res Cardiol*. 2001;96:364 –368.
31. Vinge LE, Oie E, Andersson Y, Grogaard HK, Andersen G, Attramadal H. Myocardial distribution and regulation of GRK and beta-arrestin isoforms in congestive heart failure in rats. *Am J Physiol Heart Circ Physiol*. 2001;281:H2490–H2499.
32. Gros R, Benovic JL, Tan CM, Feldman RD. G-protein-coupled receptor kinase activity is increased in hypertension. *J Clin Invest*. 1997;99:2087–2093.
33. Choi DJ, Koch WJ, Hunter JJ, Rockman HA. Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy in increased beta-adrenergic receptor kinase. *J Biol Chem*. 1997;272:17223–17229.
34. Bonita RE, Raake PW, Otis NJ, Chuprun JK, Spivack T, Dasgupta A, Whellan DJ, Mather PJ, Koch WJ. Dynamic changes in lymphocyte GRK2 levels in cardiac transplant patients: a biomarker for left ventricular function. *Clin Transl Sci*. 2010;3:14 –18.
35. Martini JS, Raake P, Vinge LE, DeGeorge B Jr, Chuprun JK, Harris DM, Gao E, Eckhart AD, Pitcher JA, Koch WJ. Uncovering G protein- coupled receptor kinase-5 as a histone

deacetylase kinase in the nucleus of cardiomyocytes. *Proc Natl Acad Sci U S A*. 2008;105:12457–12462.

36. Gainetdinov RR, Bohn LM, Walker JK, Laporte SA, Macrae AD, Caron MG, Lefkowitz RJ, Premont RT. Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron*. 1999;24:1029 –1036.
37. Walker JK, Gainetdinov RR, Feldman DS, McFawn PK, Caron MG, Lefkowitz RJ, Premont RT, Fisher JT. G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation. *Am J Physiol Lung Cell Mol Physiol*. 2004;286:L312–L319.
38. Sorriento D, Santulli G, Fusco A, Anastasio A, Trimarco B, Iaccarino G. Intracardiac injection of AdGRK5-NT reduces left ventricular hypertrophy by inhibiting NF-kappaB-dependent hypertrophic gene expression. *Hypertension*. 2010;56:696 –704.
39. Liggett SB, Cresci S, Kelly RJ, Syed FM, Matkovich SJ, Hahn HS, Diwan A, *et al.* A GRK5 polymorphism that inhibits beta-adrenergic receptor signaling is protective in heart failure. Nat Med 2008;14:510-517
40. Benovic, J. L. Purification and characterization of β-adrenergic receptor kinase. Methods Enzymol. 1991;200:351−362.
41. Tesmer JJ, Tesmer VM, Lodowski DT, Steinhagen H, and Huber J. Structure of human G protein-coupled receptor kinase 2 in complex with the kinase inhibitor balanol. *J Med Chem* 2010;53:1867–1870.
42. Thal, D. M., Yeow, R. Y., Schoenau, C., Huber, J., and Tesmer, J. J. G. Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. Mol. Pharmacol. 2011;80:294−303.

43. Takeda Pharmaceutical Company Limited, Ikeda, S., Kaneko, M., and Fujiwara, S. (2007) Cardiotonic agent comprising GRK inhibitor, World Patent WO2007034846.

44. Mayer, G., Wulffen, B., Huber, C., Brockmann, J., Flicke, B., Neumann, L., Hafenbradl, D., Klebl, B. M., Lohse, M. J., Krasel, C., and Blind, M. An RNA molecule that specifically inhibits G protein-coupled receptor kinase 2 in vitro. RNA 2008;14:524−534.

45. Theis, M.G. *et al*. Discriminatory aptamer reveals serum response element transcription regulated by cytohesin-2. Proc. Natl. Acad. Sci. USA 101 (suppl. 31): 11221–11226 (2004).

46. Blind, M., Kolanus, W. & Famulok, M. Cytoplasmic RNA modulators of an inside-out signal-transduction cascade. Proc. Natl. Acad. Sci. USA 96 (suppl. 7): 3606–3610 (1999).

47. Shi, H., Hoffman, B.E. & Lis, J.T. RNA aptamers as effective protein antagonists in a multicellular organism. Proc. Natl. Acad. Sci. USA 96 (suppl. 18): 10033–10038 (1999).

48. Hafner, M., Vianini, E., Albertoni, B., Marchetti, L., Grune, I., Gloeckner, C., and Famulok, M. Displacement of protein-bound aptamers with small molecules screened by fluorescence polarization. Nat. Protoc. 2008;3:579−587.

49. Thal, D.M., Homan, K.T., Chen, J., Wu, E.K., Hinkle, P.M., Huang, Z.M., Chuprun, J.K., Song, J., Gao, E., Cheung, J.C., Sklar, L., Koch, W.J., & Tesmer, J.J.G. Paroxetine is a Direct Inhibitor of G Protein-Coupled Receptor Kinase 2 and Increases Myocardial Contractility. *ACS Chem. Bio.* 2012;7:1830-1839.

50. Shankaranarayanan, A., Thal, D. M., Tesmer, V. M., Roman, D. L., Neubig, R. R., Kozasa, T., and Tesmer, J. J. G. Assembly of high order Gαq-effector complexes with RGS proteins. J. Biol. Chem. 2008;283:34923−34934.

51. Stryer, L. The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. J. Mol. Biol. 1965;13:482-495.
52. Cohen, P. Protein kinases–the major drug targets of the twenty-first century? Nat. Rev. Drug Discovery 2002;1:309−315.
53. Hjalmarson A, Goldstein S, Fagerberg B et al. Effects of controlled-release metoprolol on total mortality, hospitalizations, and well-being in patients with heart failure: the Metoprolol CR/XL Randomized Intervention Trial in congestive heart failure (MERIT-HF). MERIT-HF Study Group. *JAMA.* 2000:283 (10): 1295–1302.
54. "Beta-Adrenoceptor Antagonists (Beta-Blockers)." *CV Pharmacology*, 8 Feb. 2010. Web. 07 Dec. 2012.
55. Bourin, M., and Chue, P. Paroxetine: a review. CNS Drug Rev. 2001;7:25−47.

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