Screening and Characterization of a Pan-GTPase Inhibitor

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SCREENING AND CHARACTERIZATION OF
A PAN-GTPASE INHIBITOR

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SCREENING AND CHARACTERIZATION OF A PAN-GTPASE INHIBITOR

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ABSTRACT

Abnormal functioning of small GTPases is implicated in a variety of diseases, ranging from neurological and developmental diseases to cancer. In fact, mutant GTPases are found in up to 30% of cancers. Thus, small GTPases are a highly relevant target in drug discovery and development. High-throughput targeted screening of small molecules is the most productive method of discovering compounds that can give insights into drug development. This thesis describes improvements made to a high-throughput GTPase-targeted screening method to minimize confounding systematic error. It also describes the follow-up characterization of a compound that was identified in a high-throughput screen. The compound under investigation, PR-619, was shown to be a pan-GTPase inhibitor that competitively inhibits guanine nucleotide binding in a panel of sixteen members of the Ras superfamily of small GTPases. Additionally, PR-619 was demonstrated to inhibit GTPase-effector interaction and to produce effects in cellular studies.
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CHAPTER 1: Introduction

1.1. Overview and Project Aims

Mutant GTPases have been associated with several human diseases and carcinomas \(^1\text{-}^{13}\). Because of their involvement in cell signaling, aberrant activity in GTPases can lead to excessive cell proliferation, motility, and other hallmarks of cancer. This, in addition to their ubiquitous presence in nearly every cellular process, has made GTPases important targets for therapeutics and molecular probes.

Dr. Sklar and collaborators have previously demonstrated the utility of a microsphere bead-based high-throughput flow cytometry assay for the identification of GTPase inhibitors and activators from screens of several molecular libraries \(^14\text{-}^{20}\). The assay can be used for initial identification of GTPase-active compounds, followed by rapid dose response generation and real-time kinetic studies of identified compounds. The assay has also been adapted into a multiplex form in which multiple GTPases can be analyzed at once, further increasing the rate at which compound libraries can be screened. However, there is evidence that the multiplex assay allows cross-contamination of GTPases, leading to confounding systematic error.

The first of the two primary objectives addressed in this thesis was to improve the existing multiplex screen by preventing cross-contamination of proteins between beads. This objective was met by increasing site occupancy on the microsphere beads, thereby minimizing the available sites where cross-contaminating proteins may adhere after dissociating from the microsphere to which they were originally bound. These
adjustments were applied in a compound library screen using the improved multiplexed system.

The second objective of this work was to characterize a compound that had been identified as a pan-GTPase inhibitor in a previous compound library screen. The inhibitor under investigation, PR-619, was characterized for its mechanism of inhibition, inhibition of effector binding, and intracellular GTPase inhibitory activity. Collectively, these results present PR-619 as a novel competitive inhibitor of a broad spectrum of Ras superfamily GTPases with potential for use as a molecular probe.

1.2. Background

1.2.1. GTPases

GTPases are intracellular enzymes loosely anchored to the plasma membrane that act as binary molecular switches for a variety of cellular processes. GTPases can be split into two broad classes: heterotrimeric G-proteins, and monomeric small GTPases \(^{1,2,21,22}\). Both classes transmit signals from cell membrane receptors to intracellular targets. All GTPases, regardless of class, perform the same function: to bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Downstream effects that are regulated by signaling processes involving GTPases include activation of transcription factors, cytoskeletal rearrangement via polymerization and depolymerization, and other diverse responses.

Heterotrimeric G-proteins are associated with seven-transmembrane receptors (also called G-protein coupled receptors, GPCRs). GPCR interaction with an extracellular
ligand induces a conformational change in the receptor that subsequently allows for activation of the heterotrimeric G-protein, which in turn initiates signal transduction.

Small GTPases are similar to heterotrimeric G proteins in their association with the cell membrane and involvement in signal transduction. When bound to GTP, the GTPase is in the active state and has an increased affinity for effectors that carry out signaling or regulate cellular processes \(^{1,7,21,22}\). Conversely, GDP-bound GTPases are inactive. These states of activity relate to the ability of the GTPase to recruit effector proteins and carry out downstream signaling: effectors can only interact with GTPases when the GTPase is in the active state \(^{8,9}\). Wild-type GTPases rest in the inactive state and are activated in response to upstream signaling, but oncogenic and other disease-causing mutations can leave GTPases constitutively active or inactive. All GTPases cycle between the inactive and active states primarily by the mediation of two classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). When signal transduction begins, GEFs remove the GDP from the nucleotide binding cleft, and the high proportion of free GTP to GDP in the cell favors GTP loading onto the GTPase \(^{2,10,23}\). GTP binding induces conformational changes to the Switch 1 and 2

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**Figure 1.1.** Schematic depicting GAP-mediated hydrolysis of GTP and GEF-mediated replacement of GDP for GTP.
regions of the GTPase, allowing it to bind to other regulatory and effector proteins to transduce a signal (Figure 1.1). Because the intrinsic hydrolytic activity of GTPases is typically low, GAPs, which enhance the enzymatic activity of the GTPase 100-fold, are necessary to stimulate GTP hydrolysis to GDP, thus bringing the GTPase to its inactive state and terminating the GTPase signal.

The Ras superfamily of small GTPases can be divided into five main subfamilies on the basis of sequence and function: Ras (Rat sarcoma), Rho (Ras homolog), Ran (Ras-like nuclear protein), Rab (Ras-like protein in brain), and Arf (ADP-ribosylation factor). All members of the Ras family share a common biochemical mechanism and a set of conserved residues in the nucleotide binding motif that make up the G domain, which is responsible for both nucleotide binding and effector interaction. Of the five subfamilies, the Ras subfamily, comprised of 36 members, is the most well-researched because of its role in human oncogenesis. Ras GTPases are primarily implicated in the regulation of gene expression and cell proliferation, differentiation, morphology, survival, and apoptosis. The Rho subfamily, comprised of 20 members, is responsible for the regulation of extracellular stimulus networks that regulate actin organization, cell cycle progression, and gene expression. Notable members of the Rho family include RhoA, Rac1, and CDC42. The Rab subfamily is the largest branch of the Ras superfamily with 61 members. Rab proteins integrate signals relating to vesicular transport and protein trafficking between organelles. The Arf family consists of 30 members that are involved in the regulation of vesicular transport. The Ran family consists of only one protein but is the single most abundant small GTPase in the cell. It is involved in nuclear transport of RNA and proteins.
Each subfamily of GTPases has its own set of GEFs and GAPs, which are structurally distinct but functionally similar across families. The Rho and Rab families have a third family of activity regulators called guanine nucleotide dissociation inhibitors (GDIs), which prevent GDP dissociation from GTPases by binding and sequestering the GTPase. Additionally, each subfamily has a unique set of effector proteins that it can activate.

Because of the crucial roles that small GTPases play in cell signaling and other functions, missense mutants can lead to a variety of diseases. Ras subfamily mutants, in particular, are key players in both tumorigenesis and tumor maintenance, and as a result of their high rate of occurrence in human cancers have been the most well-studied family of small GTPases. Ras subfamily mutations are found in an estimated 15-30% of human cancers, with an even larger incidence rate in specific types of cancer. Most notably, KRAS mutations are found in 98% of pancreatic ductal adenocarcinomas, 15-50% of lung cancers, and 50% of breast cancers. In fact, mutations in most GTPase subfamilies are associated with neoplastic transformation, and individual subfamilies have additional associations with other disorders in humans. For example, RAS mutations are associated with a spectrum of developmental disorders, RHO mutations with immunodeficiency syndromes, and RAB mutations with inherited neuropathies and Alzheimer’s Disease. Because of the prevalence of GTPase gene mutations in disease, small GTPases have been an aspirational therapeutic target for several decades. However, there are still no clinically available drugs that target mutant GTPases, although several are currently in clinical or preclinical studies.
1.2.2. Review of Related Literature

Historically, small GTPases have been considered “undruggable” for their smooth and shallow binding sites and their high affinity for guanine nucleotides, although recent research has proven otherwise. One of the earliest GTPase inhibition studies attempted to prevent Ras farnesylation, a posttranslational modification that allows for membrane localization. Unfortunately, farnesyltransferase inhibitors failed in clinical trials due to toxicity. More recent approaches include targeting other binding sites on GTPases to prevent interaction with effectors, such as kinases, and regulators, such as GEFs, or direct inhibition of the effectors and regulators themselves. However, inhibition of effector and regulatory proteins instead of the direct GTPase inhibition can lead to unexpected consequences: studies on farnesylation inhibition, for example, were successful in biochemical systems but were unsuccessful in vivo because of the existence of a previously undiscovered secondary membrane localization system.

Several direct inhibitors of GTPase-nucleotide binding have been discovered. GTPase targeting can occur directly, as both the drug and the nucleotide compete to bind in the same site, or allosterically, as the drug binds to another site on the GTPase to prevent or enhance GTPase activation. None of the GTPase-targeted drugs that have reached preclinical or clinical trials are direct competitors for the nucleotide binding cleft, possibly due to the long-standing belief that no inhibitory drug could compete with the high affinity of GTPase to nucleotide and the high nucleotide concentration in the cell. Nonetheless, GTP-competitive molecules that directly inhibit nucleotide binding have been discovered and used as molecular probes.
1.2.3. Methods of GTPase Interrogation

Methods for the development and discovery of GTPase-inhibiting compounds include *in silico* screening, usually in tandem with structure-based drug design \(^{33,38,39}\), and *in vitro* screening of drugs to inhibit the activity of GTPases, their effectors, or their regulatory proteins. *In vitro* characterization is typically performed using solution-based biochemical assays of purified GTPases (and sometimes associated regulatory proteins and effectors) to monitor changes to fluorescent molecules as a result of GTPase activity \(^{33,40,41}\). However, biochemical assays generally require large quantities of purified GTPases, as opposed to biochemical assays on kinases and GTPase effectors which require up to a 1000-fold smaller concentration than small GTPase assays \(^{33}\). This difference arises from the low enzymatic activity of GTPases compared to kinases and GTPase effectors \(^{33}\) and makes direct GTPase inhibition assays costly.

Methods for direct measurement of GTPase-nucleotide binding include quantitative assays, such as ligand overlay blotting, thin layer chromatography, immunoprecipitation\(^ {17}\), and real-time quantitative assays using time course-HPLC and nuclear magnetic resonance (NMR) \(^ {42,43}\). However, these assays still require very high GTPase concentrations \(^ {42}\) and are not cost-effective methods real-time GTPase activity quantification.

To overcome the hurdles to high-throughput GTPase activity quantification, Dr. Sklar and collaborators developed a bead-based assay for real-time measurement of GTPase-nucleotide binding \(^ {17}\). In this method, GTPases are bound to microsphere beads, effectively concentrating the GTPase at the bead surface and enabling analysis using flow cytometry. The GTPases are treated with a fluorescently labeled nucleotide, facilitating
detection of nucleotide-bound GTPase, which is in turn bound to the microsphere bead. This assay has been used with success in numerous chemical library screens, resulting in the identification of several GTPase inhibitors and activators. This assay is discussed in greater detail throughout this thesis.

1.3. Flow Cytometry

Flow cytometry is a laser-based technique originally developed for the analysis of single cells that has also been applied to detect microsphere beads and other particles suspended in solution. Flow cytometry is unique in that it interrogates individual cells or particles and can provide information on particle size and fluorescence, either as a result of autofluorescence or fluorescent staining. Since its development in the 1970s, flow cytometry has become a powerful and broadly applicable tool in the field of biotechnology because of its unique ability to quantify the distribution of characteristics in a population of particles.

A flow cytometer consists of three key systems: fluidics, optics, and electronics. The fluidics system retrieves the liquid sample and injects it into a stream of sheath fluid in laminar flow, resulting in hydrodynamic focusing of the sample stream. Ideally, the focused sample stream produces a single-file stream of particles, allowing for individual interrogation of each particle. Interrogation occurs as the focused sample stream flows past a laser beam directed orthogonally to the flow of the sample, and the resulting scattered or fluorescent light from each particle is detected by a series of light sensors, typically photomultipliers or photodiodes, which then convert the light signal into an electronic signal.
Upon interrogation, particles scatter visible light in both the forward and side directions. The forward-scattered light provides information about particle size, while side-scattered light can indicate particle size or cell granularity and morphology, among other qualities. The laser beam also excites fluorescent molecules in the particles, which can be used to tag individual populations of particles, allowing the populations to be separated and analyzed individually, or which can be used to tag and detect specific intracellular components within a single population. Most flow cytometers have several light sensors, each with a different range of fluorescence emission wavelengths it can detect. These individual channels enable measurement of multiplex scattering or fluorescence signals from each detected particle. Analysis of flow cytometry data usually involves selection (gating) of specific events of interest based on scatter or fluorescence levels, followed by analysis of the full multi-parametric data set associated with each gated event.

1.4. Overview of Thesis

The work described in this thesis is divided into two parts: first, the improvement of an existing high-throughput screening system for detecting small molecules with activity on small GTPases, and second, the characterization of a pan-GTPase inhibitor that was identified using a high-throughput screen. Chapter 2 describes the methods, results, and outcomes of the assay improvement process. Chapter 3 details the characterization of a previously identified pan-GTPase inhibitor, PR-619. PR-619 was characterized in biochemical assays to determine its mechanism of inhibition and ability to inhibit effector binding, and in cellular assays, where its effects on intracellular GTPases and
heterotrimeric G proteins were investigated. The final chapter of this thesis summarizes the work and proposes future directions for research.
CHAPTER 2: Multiplex Screen Improvement

2.1. Introduction

The discovery of new drugs in wet lab screening (i.e. not through computer-aided drug design) is often performed in high throughput, where several hundred or thousand chemicals, typically organized into compound libraries, are rapidly evaluated for efficacy \(^{46,47}\). These screens generally have a low hit rate, but can nonetheless be a productive method for compound or drug discovery \(^{46}\). Screening methodologies can be divided into two categories: phenotypic and target-based screening. In phenotypic screening, also known as classical pharmacology, whole cells or organisms are treated with compounds and the phenotypic effects of the compounds are observed. In target-based screening, also called reverse pharmacology, target proteins are selected prior to the screen, and compounds are assessed for efficacy on those target proteins \(^{47,48}\). There is some overlap between the two screening methodologies with targeted cell-based screening. Phenotypic identification of hits is followed by investigation into the mechanism of the drug’s efficacy, while target-based hit identification is followed by studies in cells, tissues, and whole organisms \(^{47}\).

The target-based screen described in this chapter is designed for the rapid detection of GTPase-inhibiting or -activating compounds in several GTPases at once. This involves combining several GTPases that are sequestered by species on microsphere beads. Maintaining this separation between GTPase species is crucial to this screening methodology, as cross-contamination of GTPase species could lead to confounding systematic error. This chapter details the process of improving the existing method for multiplexed screening of GTPase-inhibitory compounds to prevent such anomalies.
2.2. Bead-Bound GTPase assay

This GTPase assay uses polystyrene microsphere beads of 4 μm diameter that have been functionalized with glutathione (GSH). Each set of beads is colored with a fluorescent red dye at varying intensities, allowing for separation of individual bead populations in a multiplexed assay.\textsuperscript{14–20} (Figure 2.1, following page).

Individual bead sets of a single fluorescence intensity are coupled to fusion proteins of GTPase with glutathione S-transferase (GST). GSH is a substrate of GST, enabling adhesion of the GST fusion proteins to the GSH sites on the bead. To measure nucleotide binding of the GTPase, GTPases are treated with a guanine nucleotide conjugated to a fluorescent BODIPY-FL tag (referred to as BODIPY-GTP or BODIPY-GDP). BODIPY-FL fluoresces green, so its presence on the bead can be detected in a separate detection channel than the bead-associated red fluorescence when analyzed via flow cytometry.

Inhibition of GTPase-nucleotide binding can be quantified by the green fluorescence intensity of each bead relative to a control. BODIPY-GTP shows significant quenching when not bound to GTPase as a result of photoinduced electron transfer between BODIPY-FL and nearby guanosine molecules.\textsuperscript{49} Thus, BODIPY-nucleotide is very weakly fluorescent in solution, and there is no confounding fluorescent signal from the solution itself.

In the multiplex assay for high-throughput screening, up to nine bead sets are coupled with nine different GTPases. The beads are all combined and treated with the compound under investigation before addition of the fluorescent BODIPY nucleotide. Inhibitory compounds are expected to show reduced green fluorescence intensity on beads.
Figure 2.1. Principles of multiplexed bead-based assay. (A) Individual microsphere beads with surface GSH sites are coupled with a GST-GTPase fusion protein, adhering the protein to the bead. To detect nucleotide binding, a GTP or GDP with a fluorescent BODIPY tag is used. (B) Each microsphere bead, with varying red fluorescence intensity (FL4 channel), is coupled to a unique GTPase and treated with BODIPY-GTP. (C) Bead populations can be selected in the FL4 channel because of their varying red fluorescent intensities. (D) Then, the fluorescent intensity of BODIPY on individual bead populations is measured in the FL1 (yellow-green) channel.
compared to an untreated control, and activating compounds are expected to do the opposite.

2.3. Methods

The microsphere preparation process has been described previously 14–20. The GSH-functionalized beads were washed twice with Buffer 1 (1 mM MgCl₂, 125 mM ammonium sulfate, 1 mM DTT, 0.5 mM EDTA, 0.01% NP-40, 0.1% BSA, 20 mM HEPES pH 7.5), resuspended in the buffer, and incubated for 20-30 minutes at room temperature with gentle rotation to block nonspecific binding sites on the beads 50. The beads were then collected via centrifugation, resuspended in a solution of a GST-GTPase fusion protein, and incubated overnight at 4°C with rotation. After incubation, the beads were collected and the supernatant removed, then washed twice in Buffer 1. In a typical multiplex experiment, a bead set is included that has not been coated in GST-GTPase, leaving the GSH sites empty. This “scavenger” bead serves to collect some of the GST-GTPases that dissociate from other beads during the experiment to prevent reassociation with another bead.

2.3.1. GSH Site Occupancy

For determining the GSH site occupancy of beads, individual bead sets were combined and diluted to 1800 beads per microliter in Buffer 1, with one sample containing protein-coupled beads and another uncoated beads. A stir bar was added to each sample, followed by 100 nM GST-green fluorescent protein (GST-GFP), and the samples were incubated at room temperature with constant stirring for 25 minutes before flow cytometric reading. Percent coverage, a measure of GSH site occupancy, was calculated as
\[ \text{Coverage}_i \% = \left( 1 - \frac{MCF_{i, \text{coated}}}{MCF_{i, \text{uncoated}}} \right) \times 100\% \quad \text{Eq. 2.1} \]

where \( MCF_i \) is mean channel fluorescence for bead set \( i \), and \textit{coated} and \textit{uncoated} indicate bead sets that have been and have not been coated with GST-GTPase fusion protein, respectively.

The scavenger bead in the multiplexed GST-GFP occupancy experiments consistently yielded a percent coverage between 0\% and -20\%. Negative site occupancy would result when the ratio \( \frac{MCF_{i, \text{coated}}}{MCF_{i, \text{uncoated}}} \) is greater than 1, i.e. the scavenger bead in the sample of protein-coated beads is bound to more GST-GFP than the same bead in the sample of uncoated beads. This anomaly may be due to the high concentration of free GSH sites in the uncoated sample and the potential depletion of the GST-GFP in solution. In the GST-GTPase-coated sample, the total concentration of free GSH sites is much lower than in the sample without GST-GTPase. At an estimated 1.2\( \times 10^6 \) GSH sites per bead, the concentration of free GSH sites in the uncoated sample is approximately 3.6 nM. This is in contrast to the protein-coated sample, where the concentration of free sites is estimated at 1.4 nM using coverage data from Figure 2.4A. It is also possible that the calculated negative percent coverage is the result of density-dependent fluorescence quenching on the uncoated beads. To correct for this anomaly, the negative percent coverage is subtracted from the calculated percent coverage for each protein.

\subsection*{2.3.2. BODIPY-Nucleotide Binding Measurement}

To measure the capacity of each protein-coated bead to bind BODIPY-GTP, the bead sets were combined as described previously and diluted to 200 beads per microliter in Buffer 1. Then, either unlabeled nucleotide at 100 times the BODIPY-GTP concentration
(negative control) or additional Buffer 1 were added to the beads and the samples were incubated for 30 minutes at 4°C with rotation. After incubation, 30 μM BODIPY-GTP was added to samples. The samples were incubated for 2 hours at 4°C with rotation, and data was collected using flow cytometry.

2.3.3. Compound Screening

For multiplex compound screens, individual bead sets were combined and added to a 384-well plate containing either compound, DMSO (untreated control), or unlabeled nucleotide (negative control) in each well. After 1 hour of incubation at 4°C with gentle agitation, BODIPY-GTP was added at 30 μM and incubated for an additional 2 hours. The plate was then analyzed cytometrically.

Figure 2.2. Schematic depicting the methodology for measuring site occupancy by GST-GFP fluorescence intensity and for measuring BODIPY-GTP binding to GTPases.
2.3.4. Analysis Methods

The multiplex data can be separated into individual bead sets on the basis of red fluorescence intensity on the FL4 channel. Green BODIPY-FL or GFP are read on the FL1 channel. Thus, the bead fluorescence and nucleotide-binding fluorescence are read on different channels, so there is no effect of bead color on BODIPY-FL or GFP fluorescence measurement. Initial gating of cytometric data was performed using HyperView software.

The activity of bead-bound GTPases is quantified by the relative BODIPY fluorescence intensity of each bead. Each sample or plate of samples is accompanied by a negative control, where the GTPase beads are treated with unlabeled nucleotide in 100-fold excess of BODIPY-nucleotide. The green fluorescence intensity of the control is indicative of non-specific binding of BODIPY-nucleotide to GTPase or the bead surface, i.e. BODIPY-nucleotide binding to sites other than the GTPase nucleotide binding cleft. The net (specific) BODIPY-nucleotide fluorescence intensity is calculated

\[ MCF_{i,\text{net}} = MCF_{i,\text{compound}} - MCF_{i,\text{control}} \]  

Eq. 2.2

where \( MCF_{i,\text{compound}} \) is the MCF of bead set \( i \) treated with the compound under investigation, and \( MCF_{i,\text{control}} \) is the MCF of bead set \( i \) treated with 100-fold excess of unlabeled nucleotide. In this case, all bead sets are coated in GST-GTPase fusion protein, unlike the experiment analyzed by Eq. 2.1.
2.4. Results & Discussion

2.4.1. Assay Improvements

Traditionally, these high-throughput screens have been conducted with each bead set coupled at the same molar concentration of GTPase relative to the number of beads: 1 μM GTPase per 1,000,000 beads in a volume of 20 μM, where each bead has ~1.2x10^6 GSH sites. However, for reasons that are not clear, not all GST-GTPase fusion proteins show the same nucleotide-BODIPY fluorescence intensity or the same coverage of GSH sites on the bead (Figure 2.4A-B). Although the inconsistent fluorescence intensity of BODIPY-nucleotide between beads can be normalized by comparing the relative fluorescence intensity of compound-treated sample to the untreated control, poor GSH site coverage presents an issue in a multiplexed assay. Because kinetic equilibrium is not static on the time frame of tens of minutes, GST-GTPases are constantly dissociating from and re-associating with beads. If there are available sites on other beads, free GST-GTPases from one bead set may associate with unoccupied sites on another bead set, leading to systematic error and an inability to discern the effects of compounds on specific GTPases (Figure 2.3). This error is especially problematic in the case of compounds with selective activity on specific GTPases, where cross-contamination would appear as a

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**Figure 2.3.** Schematic of cross-contamination error in bead-based multiplex system.
Figure 2.4. Site occupancy and BODIPY fluorescence intensity. (A) Site occupancy of each GTPase-coated bead in an eight-GTPase multiplex. GST-GTPase displayed irregular site occupancy despite coupling under identical, standardized conditions (1 μM GST-GTPase, 1x10^6 beads, 20 μL volume, 18 hr coupling). Site occupancy was measured from the fluorescence of GST-GFP relative to control beads not coated with GST-GTPase as described in the text. These specific GTPases were selected for the multiplex for their relevance in carcinomas and other diseases. (B) BODIPY-GTP fluorescence of the same multiplex as figure A, also showing irregular fluorescence for each bead, presumed to indicate differences in GSH site occupancy or GTPase nucleotide binding affinity. MCF = mean channel fluorescence. (C) Site occupancy improvements made to four GTPases selected for their poor site coverage (<60%) in figure A. The x-axis label “1x” indicates standard coupling conditions, “2x” indicates a 2-fold increase in protein concentration while all other coupling parameters are maintained, etc. For all GTPases investigated, increasing protein concentration resulted in increases in site occupancy. Site occupancy greater than 60% was achieved for all four proteins investigated.
significant effect on the specific GTPase, and effects of a smaller magnitude on GTPases on which there is actually no effect.

To reduce the concentration of free GTPase, each multiplexed experiment includes a “scavenger” bead, one which has not been coupled to protein and is included as a high-availability site for dissociated GST-GTPases to bind. In multiplexed experiments, the scavenger bead shows fluorescence on the same channel as BODIPY-FL, suggesting that GST-GTPases do in fact dissociate from beads (Figure 2.4B). The presence of free GST-GTPase only presents an issue, however, if there are sufficient sites available on non-scavenger beads – in that case, free GST-GTPases may attach to the available sites, cross-contaminating proteins between beads.

Figure 2.4A shows the percentage of free sites on each bead set as measured by GST-GFP fluorescence intensity on protein-coupled beads. The threshold for adequate coverage was set to 60% to ensure that over half of the sites on the bead were occupied by the desired GTPase, and thus RhoA, Cdc42, Rac1, and the KRas Q61H mutant were selected for coverage improvement.

First, the concentration of GTPase in the bead coupling reaction was increased to push the equilibrium state toward a higher percent coverage of GSH sites, as described by the equation

\[ \theta = \frac{[GST \ GTPase]/K_d}{1 + [GST \ GTPase]/K_d} \]  

\[ Eq. 2.3 \]

where \( \theta \) is site occupancy and \( K_d \) is the equilibrium dissociation constant of GST-GSH, reported as 80 nM. This equation provides a theoretical motivation for increasing
coupling concentration with the intent to increase the value of $\theta$. Figure 2.4C shows percent coverage assessed at several coupling concentrations, where 1x represents the standard 1 $\mu$M GST-GTPase per million beads, 2x represents a 2-fold increase in protein concentration, i.e. 2 $\mu$M GST-GTPase per million beads, and so on. These results show a clear relationship between concentration and site occupancy: increased coupling concentration results in increased site occupancy.

To determine whether the improvements to site occupancy were a result of reactant quantity or density, the volume of the coupling milieu was reduced 4-fold while the quantities of protein and beads were kept constant, thereby reducing the reaction volume to increase reactant concentration without increasing reactant quantity. Reducing the reaction volume had no apparent effect on site occupancy (data in Appendix B).

The standard multiplex assay protocol was changed to reflect the results in Figure 2.4C. RhoA, Cdc42, and Rac1 were coupled at 3 times the standard protein concentration, KRas Q61H at 4 times the standard, and all other proteins were coupled at the standard coupling conditions.

2.4.2. Screening

In an application of the improvements made to the multiplex bead screening process, over 1,200 small molecules from the Prestwick Chemical Library were screened for GTPase inhibitory or activating properties using the previously described methods to improve bead site occupancy. In these screens, a compound identified as a pan-GTPase inhibitor from a previous screen, PR-619, was used as a negative control. Dose response data from the previous screens that identified PR-619 as an inhibitory compound can be found in Appendix A.
2.5. Chapter Summary and Conclusions

This chapter outlined improvements to a preexisting assay to minimize systematic error that renders the multiplex methodology ineffective. Increasing the concentration of protein present in the bead coupling milieu resulted in improved site occupancy of GSH beads, which reduces the available GSH sites for dissociated proteins to bind in a multiplex assay.

As bead coverage drops, it is possible that a variety of free GTPases from other beads may associate with the low-coverage bead, leading to increased response resulting from non-target GTPases on the incorrect bead. The results presented in this section show successful improvement of GSH site occupancy to ensure that all protein-coated beads show over 60% coverage.

However, this does not completely eliminate the possibility of protein cross-contamination between beads. Instead, it reduces the magnitude of error that results from cross-contamination, since fewer available sites on the bead implies less adhesion of dissociated GTPases to the bead. Thus, the results from the multiplex still include a margin of error that is difficult to isolate or account for. Nonetheless, future multiplex results will still provide qualitative information regarding the inhibitory or activating effects of compounds. It is also understood that the quantity of free GTPase in solution is significantly lower than the concentration of bead-bound GTPase, which again reduces the possibility of cross-contamination so severe that the results are uninterpretable.
CHAPTER 3: PR-619 Characterization

3.1. Introduction

PR-619 (3,5 dithiocyanatopyridine-2,6-diamine) is a reversible broad-spectrum deubiquitinase inhibitor that has been used to study the involvement of deubiquitinating enzymes in various cellular processes. It has also been reported to be a potent DNA topoisomerase II poison \(^5\).

PR-619 was identified as a pan-GTPase inhibitor in the Selleckchem L1700 Bioactive Compound Library with an EC50 between \(~2\) and \(~35\) μM with inhibition demonstrated on eight KRas GTPases and nine non-KRas GTPases (see Appendix A). In target-based screening, it is typical for hit compounds to be characterized in follow-up studies.

This chapter describes the follow-up characterization of PR-619. First, PR-619 was tested for autofluorescence and fluorescence quenching to confirm that the existing dose response data was not the result of an artifact. Then, a biochemical assay was conducted to determine the compound’s mechanism of GTPase inhibition, followed by an assessment of the compound’s ability to inhibit GTPase-effector interaction. Finally, PR-619 was used in two cell-based assays to assess cell membrane permeability, efficacy in cells, and off-target cellular effects.

3.2. Methods

3.2.1. Solution-Based Quenching Investigation

To ensure that the observed GTPase inhibitory dose response was not an effect of fluorescence quenching by PR-619, a solution-based biochemical assay was devised. Solutions of 300 nM BODIPY-GTP, 50 μM PR-619, and a combination of both 300 nM
BODIPY-GTP and 50 μM PR-619 were prepared in two buffers that were used for biochemical bead-based studies of the compound: Buffer 1, described previously, and NP-HPSE buffer (20 mM NaCl, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.01% NP-40, 0.1% BSA, 30 nM HEPES pH 7.5).

The solutions were loaded into a 384-well plate and analyzed using a plate reader with a fluorescence excitation wavelength at 488nm and emission at 520nm. Data were analyzed as the average fluorescence intensity of replicate wells.

3.2.2. Bead-Based Kinetics

Rac1-covered beads were prepared as described previously. For kinetics experiments, the beads were diluted to 100-300 per microliter of NP-HPSE buffer and agitated with a magnetic stir bar throughout the assay. After beginning data acquisition, 30 nM BODIPY-GTP or BODIPY-GDP was added to the sample tube, followed by addition of compound or DMSO several minutes later. Ten minutes after compound addition, unlabeled GTP or GDP was added.

The dissociation rates of BODIPY-nucleotide were calculated over the time period after the addition of PR-619 or unlabeled nucleotide using a one-phase exponential decay model. To calculate dissociation rate after compound addition, the kinetic data was normalized to the untreated control (where only DMSO was added) by dividing the two data sets, and the compound-mediated dissociation constant was calculated by fitting to a one-phase exponential decay model using GraphPad Prism version 8.4.0 for Windows.

\[ Y = (Y_0 - Plateau) \times \exp (-K \times t) + Plateau \]  

Eq. 2.4
Here, $Y$ is the signal, in this case mean channel fluorescence, $Y_0$ is the signal at time $t=0$, the plateau is the signal at infinite time, $K$ is the rate constant, and $t$ is time. The start time for exponential decay was set as the time of compound addition in the kinetic assay.

3.2.3. Cell-Free G-Trap Assay

This assay exploits the selective binding of GTPase effectors to active GTPases in order to “trap” active GTPase on the surface of microsphere beads. The beads are coated with an effector-GST fusion protein, and then treated with a solution of GTPase specific to that effector. Only active GTPases will bind to the effectors on the bead, allowing for flow cytometric analysis of the active GTPase in the solution.

Simons and Buranda et al.\textsuperscript{50,53,54} have previously published a version of this assay that uses cell lysates as the GTPase source and anti-GTPase antibodies for fluorescent labeling. The present study demonstrates this assay in a cell-free system to detect displacement of fluorescent BODIPY-GTP or BODIPY-GDP from the guanine nucleotide binding site and inhibition of GTPase-effector interaction as a result of PR-619 treatment.

Prior to coupling of effector to beads, GSH microsphere beads were washed twice in the intracellular mimic HPSMT buffer (30 mM HEPES, pH 7.4, 140 mM KCl, 12 mM NaCl, 0.8 mM MgCl2, 0.01% Tween-20)\textsuperscript{50}. The buffer was then replaced with HPSMT blocking buffer (HPSMT with the addition of 0.1% BSA) and incubated at room temperature with gentle rotation for 20 minutes to block non-specific binding sites on the beads\textsuperscript{50}. The buffer was then removed and replaced with an effector protein solution.
P21-activated kinase – p21 binding domain (PAK-PBD) was used as the effector for selective binding to active Rac1. The beads were incubated overnight in a solution of PAK-GST fusion protein in HPSMT blocking buffer at 4°C with gentle rotation. Using 30,000 beads per micromolar concentration of effector protein results in 99.6% coverage of the bead surface, which can be calculated using the relation

\[ K_d = \frac{[\text{GSH}]_e[\text{GST}]_e}{[\text{GSH GST}]_e} \]

where \([\text{GSH}]_e\) and \([\text{GST}]_e\) are the equilibrium concentrations of unoccupied GSH sites and free GST respectively, and \([\text{GSH-GST}]_e\) is the equilibrium concentration of the GSH-GST complex. The equilibrium dissociation constant \((K_d)\) of GST-GFP on GSH beads has been published as 80 nM \(^{50,51}\), and the GSH site density is estimated at \(1.2 \times 10^6\) GSH sites per bead \(^{14}\).

After overnight incubation, the beads were washed three times with HPSMT and resuspended in 100 nM Rac1 solution. Prior to introduction to the beads, soluble Rac1-GST fusion protein was incubated for 30 minutes in the presence of either 3 μM unlabeled GTP or GDP, 100 μM PR-619, or the drug vehicle DMSO. After incubation, 30 nM BODIPY-GTP or BODIPY-GDP was added to the solution and incubated for an additional 30 minutes. Finally, the Rac1 solution was combined with the PAK-coated beads and incubated for 2 hours at 4°C with rotation, then analyzed using flow cytometry.

### 3.2.4. VLA-4 Integrin Activation

This assay has been published previously \(^{37,55-57}\). U937 myeloid cells stably transfected with a non-desensitizing formyl peptide receptor (FPR) (U937 ΔST cells, henceforth...
referred to as U937 cells) were cultured to a density of 500,000-700,000 cells/mL in RPMI medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. For each assay, 1 mL of cells was transferred to a test tube and stirred gently with a magnetic stir bar for the duration of the assay.

30 seconds after beginning kinetic cytometric data collection, 25 nM LDV-FITC, an integrin ligand coupled to a fluorescent tag, was added as an indicator of integrin activation state; LDV contains an amino acid fragment of fibronectin and binds to VLA-4 with high affinity. After the FITC fluorescence intensity had stabilized, 100 nM N-Formyl-methionyl-leucyl-phenylalanyl-phenylalanine (fMLFF) was added as a ligand for the formyl peptide receptor (FPR), which initiates a signaling pathway to induce the conformational change in VLA-4 from a low affinity to high affinity state. After 4 minutes of further data acquisition, either the drug under investigation or the drug vehicle DMSO was added, followed by the addition of 1 μM unlabeled LDV two minutes later. Previous publications that did not investigate LDV dissociation rate suggest maximum integrin deactivation approximately 100 seconds after compound addition, informing the timed addition of unlabeled LDV in the present assay at two minutes after compound addition. The total volume percent of DMSO in cell solution was kept at or below 1%.

Kinetic data was acquired using flow cytometry and analyzed using HyperView and GraphPad Prism. The LDV-FITC dissociation rates after LDV addition were analyzed as two-phase exponential dissociation, with the fast and slow dissociation rates set at 0.06 s\(^{-1}\) and 0.01 s\(^{-1}\), respectively, in accordance with previously published data. This analysis was performed using GraphPad Prism version 8.4.0 for Windows. Analysis yields a
percentage of fast dissociation, which can be correlated with the percentage of low affinity integrins on the cell surface.

### 3.2.5. Solubility Studies

Solubility of the compounds used in the integrin activation assay was measured using flow cytometry. Warm RPMI in a test tube with a stir bar was placed under the sample probe of a flow cytometer. After beginning data acquisition, the compound solution in DMSO was added to bring the final concentration of compound to the concentration to be used in the VLA-4 assay. Compounds that precipitated out of solution caused an increase in events recorded by the cytometer. The volume percent of DMSO-compound solution in the RPMI sample was kept at 0.7% to maximize percent DMSO in the assay sample (and thereby maximize solubility) while keeping the total volume percent of DMSO in the VLA assay below 1% (after the addition of 1 μL each of LDV-FITC, fMLFF, and LDV in DMSO).

### 3.2.6. Calcium Mobilization

This assay measures the influx of calcium into the cytosol from endosolic stores as a result of ligand binding to the heterotrimeric G-protein-coupled FPR\(^{60-62}\) and has been reported previously\(^ {61}\).

U937 ΔST cells were cultured to approximately \(1 \times 10^6\) cells/mL as described previously. For each assay, \(5 \times 10^5\) cells were removed, collected via centrifugation, rinsed in warm phosphate-buffered saline (PBS), and finally resuspended in warm PBS. The fluorescent \(\text{Ca}^{2+}\) indicator Fluo4 was added at 21 nM and cells were incubated at 37°C for 30 minutes while protected from light. After incubation, the cells were washed twice with PBS and resuspended in warm RPMI without BSA or antibiotics. \(5 \times 10^5\) cells were transferred to a
test tube for each assay, and the bulk cell solution was kept at 37°C protected from light until use.

Shortly after beginning data acquisition, the compound under investigation or the delivery vehicle was added. The compound was added to each 500 μL sample as 0.5 μL of 50 mM PR-619 in DMSO to each 500 μL sample. Several minutes later, fMLFF was added at varying concentrations and the signal was allowed to return to baseline before ending data acquisition. As a positive control, a sample of cells was treated with 4 μM thapsigargin, which raises cytosolic calcium by inhibiting Ca²⁺ reuptake into the endoplasmic reticulum. The thapsigargin control was measured both at the beginning and end of each assay to record the decrease in cell responsiveness over time.

3.3. Results

3.3.1. PR-619 Fluorescence Artifacts

A solution-based assay was conducted to measure the autofluorescence and BODIPY-nucleotide fluorescence quenching of PR-619. Figure 3.1 (following page) shows the fluorescent effects of PR-619 in two buffers used in the bead-based biochemical assays described in this work. The compound produced no autofluorescence and no significant reduction in BODIPY-GTP fluorescence. Thus, the inhibitory effects of PR-619 observed in biochemical studies are likely not an effect of fluorescence quenching, and the results in the studies described in this section are not the result of autofluorescence.
3.3.2. Nucleotide Dissociation Kinetics

In order to investigate the inhibition mechanism of PR-619 on small GTPases, Rac1 was chosen as a model GTPase for its availability and its relatively high affinity to BODIPY-nucleotide (see Figure 2.4B).

In Figures 3.2A-B, treatment with 100 μM PR-619 results in a decrease in fluorescence intensity of both BODIPY-GTP and -GDP-treated Rac1 beads compared to the DMSO control. The addition of unlabeled nucleotide several minutes later results in a further decrease in fluorescence intensity.

To account for the non-constant response observed after the addition of DMSO, the PR-619-treated dataset was normalized to the control dataset, and the PR-619-induced dissociation constant (K_{off}) of BODIPY-nucleotide was calculated (Figure 3.2C-D). The
Figure 3.2. Nucleotide dissociation kinetics on Rac1-coated beads. (A) Kinetic data for the nucleotide dissociation experiment. Fluorescent BODIPY-GTP is added at t=30s, followed by DMSO or a solution of PR-619 in DMSO at t=600s. Addition of PR-619 induces nucleotide dissociation, observed as a decrease in fluorescence intensity. Unlabeled GTP is added at t=1200s to competitively induce BODIPY-GTP dissociation from the GTPase. (B) The same kinetic experiment as in figure A, but with BODIPY-GDP and unlabeled GDP. (C) Kinetic data for the PR-619-treated samples, normalized to the DMSO-treated sample, from t=60s to t=1200s. Black overlying curves represent the one-phase dissociation model applied to the system to calculate dissociation constant. (D) Normalized kinetic data for the GDP system. (E) BODIPY-GTP dissociation and (F) BODIPY-GDP dissociation after the addition of unlabeled nucleotide in the DMSO-treated sample. (G) Mean dissociation rate constants for BODIPY-GTP dissociation induced by GTP and PR-619, showing an insignificant difference between means. Two-tailed unpaired t-test, α=0.05, p=0.12, n=4. Error bars are ±SEM. (H) Mean dissociation rate constants for BODIPY-GDP, showing insignificant difference between GTP and PR-619. Two-tailed unpaired t-test, α=0.05, p=0.18, n=2. Error bars are ±SEM. (I) Structure of PR-619.
BODIPY-nucleotide $K_{\text{off}}$ after the addition of unlabeled nucleotide in 100-fold excess of BODIPY-nucleotide was calculated from the DMSO-treated datasets (Figure 3.2E-F).

Analysis of the BODIPY-nucleotide dissociation kinetics yields two mean dissociation constants of BODIPY-nucleotide, one as a result of treatment with PR-619 and the other of treatment with unlabeled nucleotide (Figure 3.2G-H). Notably, the mean dissociation constant was not significantly different between PR-619-treated and DMSO-treated Rac1 beads in both the GTP and GDP systems. This indicates that the PR-619 and guanine nucleotide have the same mechanism of BODIPY-nucleotide displacement $^{34}$, signifying that PR-619 is a competitive inhibitor of the guanine nucleotide binding site.

### 3.3.3. Effector Interaction

To investigate the ability of PR-619 to inhibit GTPase-effector interaction, PAK-coated beads were treated with GST-Rac1 that had been previously treated with either PR-619, unlabeled nucleotide, or DMSO, then with 30 nM BODIPY-nucleotide. Because GTPase effectors can only bind GTPases in the active state, the PAK-coated bead assay “traps” active GTPases from solution – hence the name G-trap. In this study, GTPases are activated with fluorescently labeled nucleotide, allowing for detection of PAK-bound active GTPases. This is in contrast to previous reports of this assay, which use fluorescent antibodies $^{19,50,53,54}$.

Figure 3.3 shows the inhibitory effect of PR-619 on effector binding as a reduction in fluorescence intensity compared to the DMSO-treated control in the GTP system. However, the bead fluorescence intensity after PR-619 treatment is greater than the fluorescence intensity of the negative control, which was treated with unlabeled nucleotide. This indicates that PR-619 at 100 μM only partially inhibits nucleotide
In the GDP system, the DMSO-treated control exhibits non-zero fluorescence due to the use of Rac1-GST protein; since BODIPY-GDP-conjugated Rac1-GST itself is able to bind to unoccupied sites on the bead, but is unable to bind to the effector, the observed fluorescence in this case represents bead-bound Rac1. As a result, Rac1 bound directly to the bead is indistinguishable from Rac1 bound to the effector. Thus, the results in Figure 3.3 represent some combination of both Rac1 states. Nonetheless, these results indicate that PR-619 displaces the fluorescent nucleotide and at least partially inhibits Rac1 binding to PAK-PBD. Future variations of this assay would benefit from the use of Rac1 that is not a GST fusion protein, a reagent that was not available in our laboratory at the time of this work.

Figure 3.3. Biochemical G-trap assay. PAK effector beads were treated with solutions of GST-Rac1 that had been previously incubated with either DMSO as a control, PR-619, or unlabeled nucleotide, then with BODIPY-nucleotide. In both the GTP and GDP systems, PR-619 produced a decrease in BODIPY fluorescence intensity, indicating inhibition of Rac1-effector binding. Error bars are SEM. * n=4, ** n=2.
3.3.4. Cell-Based Assays

To assess the *in vivo* efficacy of PR-619 as a small GTPase inhibitor, two assays were used, both of which stimulate the formyl peptide receptor (FPR). In the VLA-4 integrin activation assay, FPR stimulation begins a signaling cascade that is known to involve several small GTPases \(^{64-66}\). However, the FPR is a G-protein coupled receptor, meaning its stimulation and signal initiation capabilities are dependent on heterotrimeric G proteins; thus, the observed effects of PR-619 in the VLA-4 activation assay could be a result of off-target effects on heterotrimeric G proteins. To quantify these potential off-target effects, a calcium mobilization assay was used, in which FPR stimulation results in a measurable efflux of Ca\(^{2+}\) from the endoplasmic reticulum into the cytosol of the cell. Changes in calcium flux into the cytosol as a result of PR-619 treatment would suggest off-target effects.

3.3.4.1. VLA-4 Integrin Activation

To investigate the ability of PR-619 to both enter the cell membrane and inhibit small GTPases in cells, a VLA-4 integrin activation assay was conducted. The integrin affinity regulation pathway has been shown to involve the Rho-family GTPases Rac1 and Cdc42 \(^{64,65}\) and Rap GTPases \(^{66}\), as well as the G-protein-coupled FPR. Inhibition of integrin activation by PR-619 and other compounds is considered indicative of the membrane permeability and intracellular efficacy of the compound.

The VLA-4 activation pathway exploited in this assay begins with binding of formyl peptide to FPR. This initiates an inside-out signaling pathway resulting in a conformational change of the integrin that increases the integrin’s affinity for ligand. Integrins are considered to exist in several conformation-dependent states relating to
high, low, and intermediate affinity for ligand. In fluorescent integrin ligand studies, such as this study, it is not possible to discern whether all of the integrins on a cell surface are in the intermediate affinity state or if a fraction of integrins are in a high-affinity state while another fraction is low-affinity. Thus, integrin ligand dissociation is assumed to be a biphasic system for these purposes.

LDV-FITC, a fluorescently tagged VLA-4 ligand, binds to low-affinity VLA-4 with a $K_d$ of $\sim12$ nM and to high-affinity with a $K_d$ of $\sim1$-2 nM. Thus, at 25 nM LDV-FITC, both high and low affinity integrins are saturated. When unlabeled LDV is added in excess of LDV-FITC, the latter will dissociate at a greater rate from low-affinity integrins than high-affinity integrins—the lower the affinity of a ligand-receptor complex, the greater the dissociation rate constant, $k_{off}$. This principle enables kinetic measurement of the average integrin activation state. Thus, if the compound has inhibited the GTPases in the integrin affinity regulation pathway to block integrin activation, the dissociation rate of LDV-FITC will increase to reflect integrin deactivation.

This study also included CID1067700 and ML-141, two compounds that have been previously reported as GTPase inhibitors. Special attention was paid to the solubility of all compounds in this assay to maximize the potential for cell membrane permeability. Because this assay is performed on living cells, it is important to keep the total DMSO in the sample at or below 1%, as DMSO concentrations above 1% can reduce monocyte viability and responsiveness. Since the other reagents added during the assay bring the volume of DMSO in the assay sample to 0.3%, the volume of compound solution in DMSO added cannot exceed 0.7%. The assay concentrations of ML-141 and CID1067700 were selected based on previous publications, and the volume of
compound solution in DMSO was 0.7% for all compounds. The 70 μM concentration of PR-619 was chosen as a maximum soluble concentration without exceeding 1% DMSO.

Figure 3.4A shows the precipitation of compounds in DMSO when added to 37°C RPMI, measured as the change in event count recorded after compound addition. 30 μM ML-141 produces precipitate when added as 0.7% of the total sample volume, implying a dissolved concentration much lower than 30 μM. PR-619 also produces precipitate when added as 0.14% of the total sample volume to a final concentration of 70 μM, but produces minimal precipitate when the total volume of DMSO in the sample is increased to 0.7%, thus informing the decision to use 70 μM PR-619 in the VLA-4 activation study. CID1067700 shows little to no precipitation upon addition to RPMI at 50 μM.

Figure 3.4B shows the kinetic VLA-4 assay data with timed addition of reagents. In previous studies of CID1067700 and ML-141 in a VLA-4 activation assay, a decrease in fluorescence intensity was observed either transiently, as in the case of CID1067700 35, or persistently, as in the case of ML-141 19. Those effects are not observed here. This can be attributed to the use of 4 nM LDV-FITC in those studies, a concentration that is between the equilibrium dissociation constants of low and high affinity state integrins. In that system, when integrins change from the active to inactive state, LDV-FITC dissociates from the newly deactivated integrins, which presents as a loss of fluorescence. Because 25 nM is above both the equilibrium dissociation constant of high and low affinity state VLA-4, dissociation is not expected to occur when integrins change activation state. Instead, this change would be observed as an increase in the dissociation rate.
Figure 3.4. VLA-4 integrin activation assay. (A) Solubility of PR-619, ML-141 and CID1067700 in 37°C RPMI. Percentage values are the percent DMSO in the RPMI sample after addition of the DMSO-compound solution. Elevated event counts suggest compound precipitation. (B) Kinetic data from VLA-4 integrin activation assay on U937 cells. LDV-FITC, a fluorescent integrin ligand, is added at \( t=30 \)s, followed by fMLFF at \( t=120 \)s to activate integrins. DMSO or compound dissolved in DMSO is added at \( t=360 \)s, then unlabeled LDV to induce dissociation of LDV-FITC at \( t=480 \)s. Data staggered for visibility. (C) Mean percentages of fast dissociation as calculated using a two-phase dissociation model in GraphPad Prism. The means of CID1067700-treated and ML-141-treated samples were not significantly different from the DMSO-treated control (two-tailed unpaired t-test, \( \alpha=0.05 \), \( n=4 \), \( p=0.30 \) and 0.40 respectively). A statistically significant difference in mean percent fast dissociation for PR-619 treated sample was observed (\( p=0.046 \)). (D) Structure of CID1067700. (E) Structure of ML-141.
Dissociation rates were analyzed as two-phase dissociation, with the fast dissociation representing dissociation from low affinity-state integrins and slow dissociation from high affinity-state integrins. The calculated percent fast dissociation can be correlated to the relative amounts of high and low affinity integrins on the cell surface. No significant difference was observed in the percentage of fast dissociation between the DMSO control and the samples treated with ML-141 or CD1067700 (Figure 3.4C). However, the PR-619-treated shows a statistically significant decrease in the percent fast dissociation relative to the DMSO control, implying activation of cell surface integrins in response to PR-619 treatment, an unexpected effect for an inhibitory compound.

The observed insignificant changes as a result of treatment with ML-141 and CID1067700 may contradict previously published results indicating integrin deactivation as a result of compound treatment \(^{19,35}\). The principle differences between the study reported here and the previously reported study include the use of 4 μM LDV-FITC as opposed to 25 μM LDV-FITC, as previously discussed, and the special attention paid to compound solubility. The previous study on ML-141 uses a lower percentage of DMSO (total 0.6%) \(^{19}\), which would lead to lower compound solubility than what is reported here. Since the compound is sparingly soluble in water \(^{71}\), then it is possible that the intracellular concentration of the compound is not high enough to produce a significant effect on percent fast dissociation. However, this conclusion would benefit from further experimentation to determine the precise solubility of ML-141 in water and the intracellular concentration of compound. The data in this study alone do not support the hypothesis that ML-141 treatment of myeloid cells results in VLA-4 integrin inactivation.
In the case of CID1067700, previous publications reported a transient decrease in fluorescence intensity ~100s after treatment with compound, followed by a return to baseline fluorescence by ~300s after compound addition. It is possible that the CID1067700 is quickly replaced by GTP or GDP in the cell due to the high concentrations of both nucleotides relative to the intracellular concentration of the compound. In this study, unlabeled LDV was added 120s after compound, and the kinetic analysis of LDV-FITC dissociation was performed over the 7-minute span after LDV addition. Since the effects of CID1067700 were observed to be transient over a period of less than 300 seconds, the inhibitory effects are not expected to persist over the 7-minute analysis period of this study. The transience of the CID1067700 inhibitory response could explain the statistically insignificant difference in mean percent fast dissociation from the control.

The data in Figure 3.4C may also suggest that this assay method is ineffective in quantifying small changes to VLA-4 activation, since the small fluorescence changes that were observed in previous studies of ML-141 and CID1067700 are not reflected in this data.

PR-619 produced a statistically significant decrease in the percentage of fast dissociation, i.e., an increase in high affinity-state integrins on the cell surface compared to the untreated control. This suggests integrin activation, which contradicts the results of the biochemical studies on this compound. There is evidence that integrin activity regulation is mediated by a negative feedback system in which high-affinity integrins activate phosphatidylinositol 3-kinase (PI3K), which in turn activates ARAP3, a GAP for Rho and Arf GTPases, and the integrin is reverted to the inactive conformation. PI3K is a
direct effector of activated small GTPases\textsuperscript{6,73,74}, requiring interaction with activated Rho GTPases to become activated. Integrin signaling also activates Ras to initiate the MAPK and PI3K/Akt signaling pathways\textsuperscript{75}. Thus, it would follow that inactivation of Rho GTPases by PR-619 could prevent the negative feedback loop that controls integrin inactivation, leading to a higher proportion of active integrins in the PR-619-treated case than in the DMSO control. Confirming this possibility, however, would require further investigation.

3.3.4.2. Calcium Mobilization

It is established that FPR-ligand interaction induces an increase in cytosolic calcium as a result of the emptying of intracellular calcium stores\textsuperscript{76–79}. The resulting elevation in cytosolic calcium leads to the activation of kinases and other enzymes, making calcium signaling an important step in signal transduction\textsuperscript{77,78}. The chain of events from FPR stimulation to cytosolic calcium increase begins with the activation of heterotrimeric G proteins that are coupled to the formyl peptide receptor. To resolve whether the observed effect of PR-619 in the VLA-4 assay is an effect of small GTPase inhibition or off-target heterotrimeric G protein inhibition, the calcium flux in response to FPR stimulation was measured both with and without PR-619 treatment.

No significant difference was observed in the mean calcium fluorescence peak between PR-619-treated and DMSO-treated samples at several concentrations of fMLFF (Figure 3.5A). Note, however, that this observation comes with caveats which are discussed later in this section. The PR-619 response was consistently lower than the DMSO control peak response, but this can be attributed to the order in which experiments were conducted. Because the DMSO control was always analyzed before the PR-619 sample, there was a
time difference of approximately 15 minutes between the formyl peptide addition of each sample. Figure 3.5C reveals a drastic decrease in calcium responsivity as a result of thapsigargin-induced calcium efflux over the course of the experiment. The time between the initial and final thapsigargin-treated samples was less than two hours. Thus, the 10-20% decrease in peak calcium response between the DMSO-treated and PR-619-treated samples can easily be attributed to the rapid decrease in calcium responsivity. The decay
in maximum fluorescence intensity can be ascribed to the action of organic anion transporters that actively remove the fluorescent calcium indicator from the cytosol and into the extracellular space. Thus, the fMLFF-independent and statistically insignificant but consistent decrease in fluorescence intensity observed in PR-619-treated samples is considered a result of the time difference between the cytometric analysis of the DMSO-treated control and the PR-619-treated sample, and not a result of PR-619 treatment.

These results come with several caveats that render this data suggestive but inconclusive. Most importantly, this study was conducted under conditions that led to PR-619 precipitation, as shown in Figure 3.5D. PR-619 solution was added as 0.1% v/v DMSO in the sample to a final concentration of 50 μM. The precipitation of the compound under these conditions was not known at the time of experimentation. Due to unforeseen time constraints, repetition of these experiments under conditions that ensure compound solubility was not possible. Additionally, this study was conducted using 50 μM PR-619, whereas the VLA-4 activation assay used 70 μM PR-619. Even if the compound was soluble under these conditions, the lack of effects observed from PR-619 treatment may be a result of insufficient compound concentration. This makes it difficult to conclude whether the effects of PR-619 observed in the VLA-4 activation assay were related to effects on heterotrimeric G proteins.

Thus, this study alone cannot lead to the definitive conclusion that PR-619 does not affect heterotrimeric G proteins. An optimization of this study in which PR-619 is used under soluble conditions and at 70 μM would be necessary to draw this conclusion.
3.4. Chapter Summary and Conclusions

This chapter described four methods for characterizing and validating the inhibitory activity of PR-619. In a bead-based biochemical experiment, both the compound and unlabeled nucleotide showed a statistically insignificant difference in the rate of fluorescent nucleotide inhibition, suggesting that PR-619 inhibits in a competitive manner. The results of an effector-based assay suggested that PR-619 also inhibits effector binding. Both of these biochemical assays successfully demonstrate that PR-619 is an inhibitor of small GTPases.

Cell-based experiments showed mixed results. An integrin activation assay surprisingly exhibited the opposite of the expected effect of PR-619 treatment, but it is possible to rationalize this observation while maintaining the hypothesis that PR-619 is a pan-GTPase inhibitor in cells. Although PR-619 treatment did not inhibit calcium mobilization, the study itself was flawed in that PR-619 was not fully solubilized. Therefore, it is not possible to conclude whether PR-619 acts on heterotrimeric G proteins.

The results presented in this chapter would greatly benefit from further investigation. The calcium mobilization assay suffered from a rapid decrease in fluorescence intensity, as well as the precipitation of the compound under the assay conditions. To improve on this assay, cells could be treated with the organic anion transporter inhibitor probenecid to prevent the displacement of the fluorescent calcium indicator observed over the course of the experiment. Additionally, the compound could be added as a greater volume of a reduced concentration PR-619 solution in DMSO, as in the VLA-4 assay, since those conditions were shown to promote compound solubility.
One additional study to confirm the efficacy of the compound in cells might include a cell lysis G-trap assay, in which effector-coated microsphere beads are incubated in cell lysate from PR-619 treated cells. The effector “traps” active GTPases on the bead, which are then detected using labeled antibodies. Studies of this type have been previously published\textsuperscript{50,53,54} and have been used to verify efficacy of other GTPase inhibitors in cells\textsuperscript{19}. This study would also verify the cell membrane permeability of PR-619 in the myeloid cell line used in the cell-based studies presented here. Applying the G-trap assay to the other inhibitory compounds studied in the VLA-4 integrin activation assay, Ml-141 and CID1067700, would also verify whether the observed insignificant change in integrin activation is evidence of ineffective inhibitory compounds or an artifact of an insensitive assay method.
CHAPTER 4: Summary and Future Directions

4.1. Summary of Work

Aberrant activity of small GTPases has been implicated in several diseases, making GTPases a relevant focus of biomedical research. This thesis described a high-throughput screening assay to identify drugs that act on small GTPases and the follow-up studies on a compound identified from such a screen.

High-throughput screening assays must be consistent and of high quality to enable the rapid compound screening that high-throughput drug discovery entails. The work described in this thesis describes investigation into a source of systematic error in this system and the amelioration of that error. Specifically, protein displacement from beads in the multiplex system was observed as a non-zero green fluorescence on an uncoated bead. Re-association of proteins with other beads in the multiplex would lead to confounding results. The improvements made to the multiplex system demonstrated a successful increase in bead site occupancy, which in turn leads to a decreased likelihood of free-protein association with those beads. These improvements to the multiplex were implemented to screen a library of several hundred small molecules.

A pan-GTPase inhibitory compound identified in a previous screen, PR-619, was characterized for biochemical inhibitory activity and activity in cells, as is typical for hits from targeted screens. Biochemical studies of the compound’s inhibition kinetics demonstrated a competitive mechanism of action, and another biochemical study suggested inhibition of GTPase-effector binding. Cellular assays showed an unexpected effect on an integrin activation pathway. A further cell-based study attempted to
investigate whether PR-619 produces off-target effects on heterotrimeric G proteins, but flaws in the experimental methodology rendered those results inconclusive. As previously discussed, the cell-based studies presented here would greatly benefit from additional investigation to resolve the unexpected effects that were observed.

4.2. Future Directions

The improvements made to the multiplex GTPase screen have helped make the system more accurate and applicable in high throughput screening. We anticipate the use of this improved multiplex in screens where compound quantities are limited, a condition that would rule out the possibility of non-multiplex single-GTPase assays.

The data presented in Chapter 2 (Figure 2.4C) were collected in a partial multiplex. To verify the site occupancy improvements, the improved eight-protein multiplex was assembled and its site occupancy and BODIPY fluorescence intensity were measured. In Figure 4.1A, three out of the four targeted GTPases did not show the improvements in site occupancy that were observed in Figure 2.4C. Because the data in Figure 4.1 are representative of one biological replicate, it is possible that further replicates of the multiplex would reveal an improvement in site occupancy on average. Despite not showing significant improvement in bead coverage, all four of the selected GTPases did show improvements in BODIPY-nucleotide fluorescence intensity, implying a greater capacity to bind nucleotide. This may be due to the free GST that is a known contaminant of the commercially prepared Rac1, RhoA, and Cdc42 used in this multiplex\(^{81-83}\), since free GST occupying GSH sites would be indistinguishable from GST-GTPase in this non-kinetic site occupancy experiment. It is possible that increasing the concentration of protein in the coupling milieu allows a greater proportion of GST-GTPase to occupy
GSH sites, which would appear as an increased BODIPY fluorescence intensity.

Unfortunately, due to unforeseen time constraints, investigation into this phenomenon was not possible.

This multiplexed GTPase assay would greatly benefit from continued optimization and verification of the obtained results in the full multiplex. Without the opportunity for further optimization, we determined that the collective results presented in Figures 2.4C

\[ \text{Figure 4.1. Eight-protein multiplex site occupancy and BODIPY fluorescence, before and after improvements. (A) Site occupancy of the four GTPases that were selected for coverage improvement are shown in shades of blue (RhoA, Cdc42 WT, KRas Q61H, Rac1). Of the four GTPases, only KRas Q61H showed occupancy improvement in this mixed multiplex experiment. (B) BODIPY-GTP fluorescence at 30 nM. Paradoxically, all four of the selected proteins showed increases in BODIPY fluorescence, despite no improvement in percent site occupancy for three out of the four GTPases.} \]
and 4.1 were demonstrative of sufficient improvement to the multiplex system such that a chemical library could be screened, and then screened the library. Before pursuing future screens, a re-assessment of the full multiplex under the improved coupling conditions for RhoA, CDC42, Rac1, and KRas Q61H is recommended.

The results of the PR-619 characterization studies would also benefit from augmentation by further experiments. As previously mentioned, the biochemical G-trap assay would be improved by the use of Rac1 that is not a GST fusion protein. Also discussed was the use of a cell lysate-based G-trap assay. The ambiguous results produced by the VLA-4 integrin activation study could be explored by using a reduced concentration of LDV-FITC, which would allow the fluorescent molecule to dissociate from the integrin as the integrin changed in conformation. This adjustment would also permit observation of transient effects as a result of compound treatment. The calcium mobilization assay produced uninterpretable results because of the precipitation PR-619; it follows that the assay would benefit from efforts to improve PR-619 solubility.

In any screen of a small molecule library, the desired outcome is to produce compound hits with potential for use in medicine or as a molecular probe. Studies on PR-619 as a deubiquitinase inhibitor have demonstrated cytotoxicity with prolonged exposure, making it unlikely that PR-619 has potential for clinical use. However, PR-619 can be used as a control compound and molecular probe in future studies. In fact, PR-619 was used as a control inhibitory compound in a recent chemical library screen, demonstrating its potential for use as a proven GTPase inhibitor. This compound may serve as a starting point for the development of GTPase inhibitory compounds with clinical applications. Another potential application for PR-619 is as a molecular probe. Since the compound

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inhibits a broad spectrum of GTPases (see Appendix A), it could be used in cell-based studies to selectively remove GTPase activity from signaling cascades in order to further define the specific roles that GTPases play in cell signaling pathways. There are currently relatively few compounds capable of inhibiting Rab GTPases, lending this compound specific utility as a molecular probe \textsuperscript{34,35,37}. 


APPENDIX A: PR-619 Dose Response

The data in this appendix is the work of Harold Ames, Dr. Mark Haynes, and Dr. Anna Waller.

KRas GTPases

<table>
<thead>
<tr>
<th>PR-619 Dose Response</th>
<th>Normalized MCF</th>
<th>log[PR-619]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kras Q61R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras Q61L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras Q61H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras G12D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras G12C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras G12A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras G12V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kras WT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Non-Kras GTPases

<table>
<thead>
<tr>
<th>PR-619 Dose Response</th>
<th>Normalized MCF</th>
<th>log[PR-619]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RhoA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hras WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc42 L61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hras V12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rac1 L61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc42 WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rac1 WT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PR-619 inhibits BODIPY-GTP binding to several KRas mutants and several non-KRas GTPases. Samples were prepared as described in Section 2.3.3 with the use of 10 μM BODIPY-GTP. Error bars represent ±SEM. Data points are fitted with a four-parameter, variable-slope [inhibitor] vs dose response curve using GraphPad Prism 8.4.0 for Windows. The calculated EC50 values are in the table below.
<table>
<thead>
<tr>
<th>KRas GTPases</th>
<th>EC50 (M)</th>
<th>Non-KRas GTPases</th>
<th>EC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRas WT</td>
<td>1.9x10^{-5}</td>
<td>Rab7</td>
<td>2.1x10^{-5}</td>
</tr>
<tr>
<td>KRas G12V</td>
<td>2.9x10^{-5}</td>
<td>RhoA</td>
<td>2.1x10^{-6}</td>
</tr>
<tr>
<td>KRas G12A</td>
<td>3.4x10^{-5}</td>
<td>HRas WT</td>
<td>7.3x10^{-6}</td>
</tr>
<tr>
<td>KRas G12C</td>
<td>1.7x10^{-5}</td>
<td>Cdc42 L61</td>
<td>7.3x10^{-6}</td>
</tr>
<tr>
<td>KRas G12D</td>
<td>2.4x10^{-5}</td>
<td>HRas V12</td>
<td>2.2x10^{-5}</td>
</tr>
<tr>
<td>KRas Q61H</td>
<td>1.6x10^{-5}</td>
<td>Rac1 L61</td>
<td>8.8x10^{-6}</td>
</tr>
<tr>
<td>KRas Q61L</td>
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<td>Cdc42 WT</td>
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<tr>
<td>KRas Q61R</td>
<td>2.9x10^{-5}</td>
<td>Rac1 WT</td>
<td>1.1x10^{-5}</td>
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</tbody>
</table>
APPENDIX B: Site Occupancy Supplemental Data

The data below shows the results of a four-fold reduction in coupling volume from the standard multiplex preparation protocol, which calls for 1 µM GST-GTPase per 1x10⁶ beads in 20 µL volume to be coupled overnight (~18 hr). In the reduced volume case, all parameters were kept the same except the coupling volume, resulting in coupling at 4 µM GST-GTPase, 1x10⁶ beads in 5 µL volume, coupled overnight (~18 hr). The figure below shows minimal improvement in coupling for all GTPases studied.

Due to limited resources, some of the GTPases present in the eight-GTPase multiplex discussed in Chapter 2 were omitted in this experiment. The KRas WT sample prepared as in the standard multiplex protocol was lost during the sample preparation process.

In the figure below, error bars are SEM and n=2.
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