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14. ABSTRACT

Neurofibromatosis Type 1 (NF1) arises from the aberrant activation of Ras, a GTPases important controlling mitogenic potential. In an effort to control the aberrant activation of Ras to treat NF1 as well as other cancer, a high throughput screen was developed to screen for small molecule inhibitors of Ras activation by Sos1, a ubiquitous guanine nucleotide exchange factor that catalyzes the activation of Ras. Using this screen, several initial leads have been identified. Future work will define the efficacy and modes of action of these compounds to inhibit Sos1-catalyzed activation of Ras prior to more extensive quantitative structure-activity relationships.

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Introduction

A rational approach for identifying novel inhibitors of Ras

Neurofibromatosis Type 1 (NF1) is a common neurological disorder occurring in 1 out of every 40,000 individuals and is characterized by tumors of the peripheral nervous system, as well as cognitive impairment (Gottfried, Viskochil et al. 2006). Mutation of the *NF1* tumor suppressor gene plays a causal role in the progression of benign and malignant tumors typified by NF1. While NF1 can be inherited via a mutation in the *NF1* gene, about half of NF1 cases arise from *de novo* mutations in individuals without a prior family history. These mutations can range from frame shifts and missense mutations to in-frame deletions or errors in the splicing mechanism, all of which invariantly result in a loss of function phenotype. Additionally, these mutations occur in an autosomal dominant manner, where the remaining wild-type gene copy is haplo-insufficient and unable to compensate for the mutated gene.

The *NF1* gene encodes for neurofibromin, a large protein (~250 kDa) localized to the cytoplasm. Interestingly, the only domain within the sequence of neurofibromin which is recognized by current bioinformatics techniques is a Ras-GAP domain. In turn, the Ras-GAP domain is responsible for the only known biological function of neurofibromin: negative regulation of the small GTPase Ras. Ras is the prototypical member of a large group of G-proteins, termed the “Ras superfamily” or “small GTPases”, which are monomeric, approximately ~21 kDa in size, and bear significant homology to Ras. G-proteins, such as Ras, function as molecular switches that fluctuate between GTP-bound “on” and GDP-bound “off” states. G-proteins are activated via the exchange of GTP for bound GDP, a process which is catalyzed by guanine nucleotide exchange factors (GEFs). G-proteins have an intrinsic GTPase capacity which hydrolyzes GTP to yield the inactive GDP-bound state. GTPase accelerating proteins (GAPs), such as neurofibromin, increase this intrinsic hydrolysis rate of G-proteins, thereby acting as negative regulators of G-protein signaling. Ras superfamily GTPases regulate a myriad of signaling processes in the cell. In particular, Ras is a potent regulator of cellular growth in response to mitogenic stimuli. In fact, mutations in Ras rendering the protein in a persistent GTP-bound “on” state, which is insensitive to Ras-GAPs, is one of the most frequently occurring oncogenic mutations found in over 30% of human cancers. Similarly, loss of function mutations of the *NF1* gene results in persistent Ras activation, because the neurofibromin protein cannot reduce the levels of Ras-GTP.

Currently, there are few effective treatments for the tumors associated with NF1. A majority of these tumors are neurofibromas, which are benign tumors of the peripheral nerve tissue that often lead to disfigurement and a markedly reduced quality of life. Additionally, about one third of NF1 patients develop plexiform neurofibromas, which can transform into malignant and highly metastatic cancer that is typically refractory to current anti-cancer therapies. Because traditional chemotherapeutics have failed to effectively treat NF1 associated tumors, novel approaches are currently being developed to discover treatments for this devastating disorder. Moreover, because much is known regarding the underlying molecular mechanism of this disease, an emerging, yet promising strategy is to utilize a target-based approach to modulate aberrant signaling cascades mediated by loss of neurofibromin function. For example, tumor growth can be inhibited by reducing the amount of activated Ras within an NF1 tumor cell. Therefore, our main goal has been to develop novel assays to identify inhibitors of Ras which will function to reduce the amount of active, GTP-bound Ras in NF1 tumors. These assays will in turn form the basis of a future drug screening program that may ultimately lead to novel therapeutics for the treatment of NF1.

The strategy we are currently pursuing in order to identify inhibitors of Ras is to target the rate-limiting step of Ras activation, namely guanine nucleotide exchange catalyzed by Ras-GEFs, such as Sos1. This strategy will target Ras activation by preventing Ras from becoming activated by Sos1. Sos1 is a multi-domain containing protein which is a critical link between upstream mitogenic stimuli and downstream Ras activation. Sos1 activates Ras via a CDC25 domain, which is the catalytic

fragment required for guanine nucleotide exchange. ***We have developed a high-throughput screening assay which quantifies the efficiency of Sos1-mediated Ras activation in the presence of a library of compounds. We have used this assay in a pilot screen to test a set of ~2,000 diverse compounds and are currently evaluating the potency and specificity of lead compounds arising from this screen.***

Body

Task 1. Develop and validate a high throughput assay to identify compounds that inhibit guanine nucleotide exchange by Ras-GEFs.

A high-throughput assay for identifying inhibitors of Ras

In the past, most *in vitro* biochemical methods for studying the activation of Ras GTPases have relied upon radioactive forms of guanine nucleotides. Unfortunately, these methods suffer from several disadvantages including: extensive manual manipulations, limited data collection rates, the production of radioactive wastes, and discontinuous monitoring of reaction kinetics. Recent advances in spectroscopic instrumentation and the production of a variety of fluorescent analogs of guanine nucleotides have enabled fluorescence-based assays to become the preeminent method for studying many biochemical properties of Ras GTPases. These assays take advantages of spectroscopic differences between bound and unbound fluorescent nucleotide analogs to monitor guanine nucleotide exchange. Fluorophore-conjugated nucleotides, such as BODIPY-GTP (Molecular Probes), have a low quantum yield of fluorescence in solution due to intermolecular quenching by solvent and intramolecular quenching by the guanine base. However, upon binding to Ras, the fluorescence emission intensity from the fluorophore is greatly enhanced, as illustrated in Figure (**1**). Note that the addition of Sos1 is essential for this guanine nucleotide loading. Furthermore, in the presence of a compound which inhibits Ras activation, the fluorescence signal will remain low, similar to an uncatalyzed reaction.

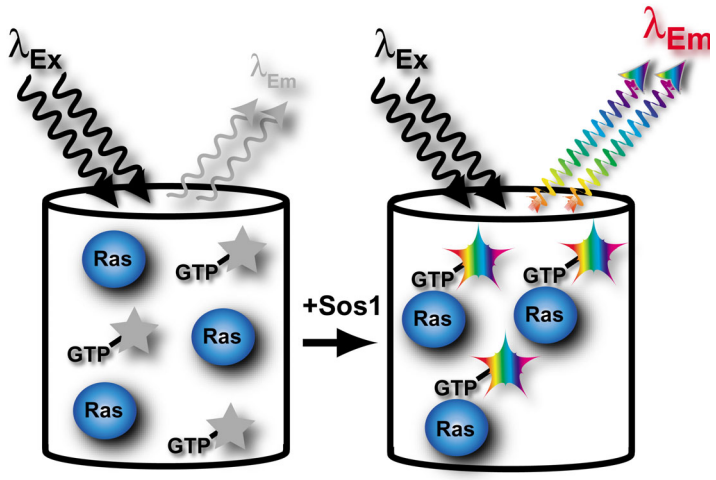

Fig. (1) The fluorescence-based guanine nucleotide exchange assay can be used to monitor activation of Ras by Sos1 using purified components in real-time. Unbound fluorophore-conjugated guanine nucleotides ("GTP", left) are quenched in solution and emit a weak fluorescence signal upon excitation. However, when Sos1 catalyzes guanine nucleotide exchange, the fluorophore-conjugated nucleotides bind to Ras, and have an increased fluorescence emission upon excitation (right).

We have developed a high-throughput version of this assay in order to identify compounds which target Ras. Figure (**2**) depicts a schematic outline of this assay with an example of experimental data from a recent pilot screen for inhibitors of Ras. Assay components, including buffer, fluorescent nucleotide, the catalytic Cdc25 domain of human Sos1, and small molecular-weight compounds from a diverse compound library are added to a microtiter plate (96-well) using liquid handling robotics (Biomek FX by Beckman Coulter) and allowed to equilibrate. Ras1 is then added to initiate the exchange process, which is followed in real-time by monitoring fluorescence intensity using a microtiter-formatted fluorimeter (Spectromax Gemini by Molecular Devices). The resulting data is fit to an exponential growth function and the observed reaction rate is calculated, which is directly proportional to the efficiency of Ras activation by Sos1. Ras GTPases have an intrinsic, spontaneous exchange rate that is independent of GEF activity. This spontaneous exchange is typically negligible but can contribute to background noise during high-throughput

screening if the exchange rate is too high. In order to minimize spontaneous exchange rates and boost the signal to noise ratio, we have empirically refined buffer components and concentrations of proteins used.

During the screening process, each 96-well plate screened contains two sets of control groups to assess assay performance: (**1**) Sos1 and Ras with DMSO but no potential small molecule inhibitor was used to define the full exchange potential of the reaction; while (**2**) DMSO with Ras only was used to quantitate the spontaneous exchange process. Data were analyzed by subtracting the initial baseline fluorescence reading at time T=0 min before the final component (Ras) was added in order to correct for fluorescence emission or quenching caused by the compound being tested in each well. The resulting data points were then fit to a single-phase exponential association function [$Y=Y_{max}\ (1-e^{(-kX)})$], where Y equals the relative fluorescence units (RFU) experimentally measured using the microtiter-formatted fluorimeter, Y_{max} equals the maximal response, k equals the observed experimental reaction rate, and X equals time in seconds. Compounds (*e.g.*, NSC 48872, NSC-154983, NSC-305821) that inhibit the observed exchange rate by greater than 75% compared to the DMSO-only control are defined as positive hits and are currently being evaluated for specificity, potency, and ease of synthesis for associated quantitative structure-activity relationships (QSAR).

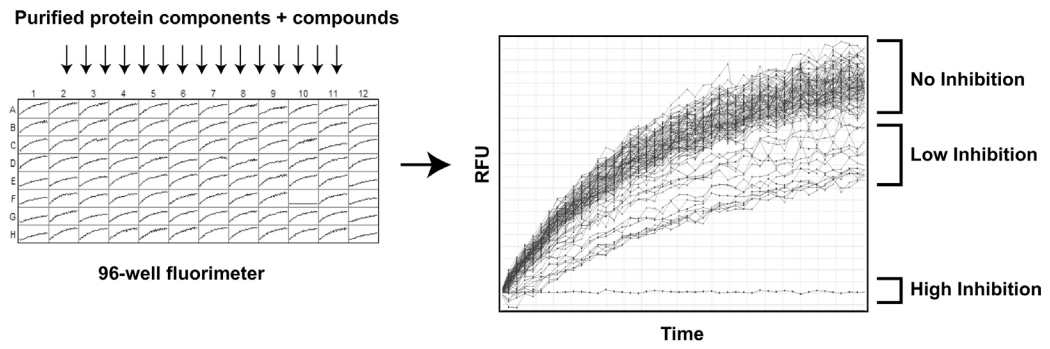

Fig. (2) Screen of ~2,000 diverse compounds for inhibitors of Sos1-mediated activation of Ras.
Purified components and compounds were added to a 96-well microtiter plate using a liquid handling robot (Biomek FX), then assayed for guanine nucleotide exchange using a microplate-formatted fluorimeter. The results of each plate (left) are graphed at right to illustrate the rates of exchange for compounds tested. Compounds that inhibit exchange have slower reaction rates than those that show no inhibition. Compounds are then scored according to % inhibition.

Protein expression and purification

Methods for purifying protein components were optimized in order to yield large quantities of both Ras and the Cdc25 domain of Sos1 (~100 mg) necessary for high-content screening. Both proteins were expressed as 6-histidine N-terminal fusions in BL21 (DE3) *E. coli.* Cells were grown in self-inducing media (adapted from Studier, F.W. (2005) *Protein Expr. Purif.*, **41**: 207-34) containing 1% (w/v) N-Z-amine AS, 0.5% (w/v) yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% (v/v) glycerol, 0.05% glucose, 0.2% α-D-lactose, and various trace metals. Bacterial cultures were grown in this high-growth, self-inducing media at 37° C until the optical density at 600 nm was equal to 1.0; the temperature was then reduced to 18° C and grown for ~18 hours. Pelleted cell cultures were then resuspended in N1 buffer (20 mM Tris pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole) and lysed with an Emulsiflex homogenizer. Lysed cells were spun for 45 min at ~50,000 x g and the resulting cleared supernatant was subjected to DNase I treatment for 30 min at 4° C. Proteins were then purified using standard chromatography using Ni-affinity columns on an FPLC. Proteins were eluted with 40% N2 buffer (N1 + 1 M imidazole) and were then subjected to gel-exclusion chromatography using an S-200 Sephacryl column (320 mL) pre-equilibrated with S-200 buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5% (v/v) glycerol, and 2 mM DTT). Protein peak fractions were determined using SDS-PAGE; fractions containing mono-dispersed protein were concentrated, flash frozen in liquid nitrogen and stored at -80° C for future

use. All Ras buffers were supplemented with 2 mM $MgCl_2$ and 30 μM GDP. After purification and concentration, all proteins were determined to be >95% pure using SDS-PAGE analysis.

96-well formatted Ras inhibitor assay

Final concentrations used during screening were as follows: 2 μM Ras, 400 nM Sos1, and 2 μM BODIPY-GTP TR (Molecular Probes) in exchange buffer consisting of 20 mM Tris pH 8.0, 300 mM $MgCl_2$, 5% (v/v) glycerol, and 2 mM DTT. A Biomek FX liquid handling robot (Beckman Coulter) was used to dispense all reagents during the screening procedure and 96-well flat-bottom black plates (Costar) were used. The final reaction volume per well was 100 μL. 96-well plates were pre-spotted with 1 μL compound or DMSO only (in control wells) resulting in a final concentration of ~10 μM compounds with 1% (v/v) DMSO. The Diversity Set of ~2,000 compounds (NCI) was used during this pilot phase of assay development, refinement, and validation. A microtiter-formatted fluorimeter (Spectromax Gemini by Molecular Devices) was used to monitor guanine nucleotide exchange in real-time using the following settings: λ_{ex}=580 nm, λ_{em}=630 nm, 46 second intervals, plate shaking between readings, auto-calibration activated, with kinetic measurements being conducted at room temperature ~25° C.

Task 2. Develop and validate a high throughput assay to identify compounds that accelerate the intrinsic GTPase of Ras.

As originally described, this assay was designed to couple the generation of inorganic phosphate with the subsequent production of phosphomolybdate which reacts with malachite green and can be monitored as absorbance at 620 nanometers Initial experiments have not been promising and suggest that Ras is unstable under these conditions. We have been exploring alternative means to carry out this task. A particularly attractive alternative method is to use phosphate binding protein which undergoes a conformational change upon binding of inorganic phosphate that can be monitored by fluorescence. Phosphate binding protein has been used under similar conditions to monitor the hydrolysis of GTP by heterotrimeric G proteins (Kimple, Jones et al. 2003).

Task 3. Execute high throughput assays developed in Tasks 1 and 2 to identify compounds that either inhibit GEF-catalyzed activation of Ras (Task 1) or accelerate the intrinsic GTPase rate of Ras (Task 2).

Extensive and on-going high throughput screening associated with Task 1 is described in more detail above. HTS associated with Task 2 has been delayed by failure to develop a robust screen.

Key Research Accomplishments

Development of a high throughput screen to identify small molecular inhibitors of Ras activation by cognate guanine nucleotide exchange factors (i.e., Sos1).

Identification of lead hits that inhibit the capacity of Sos1 to activate Ras.

Reportable Outcomes

This funding was use to support the training of Mr. Rafael Rojas, a graduate student in the Department of Pharmacology, University of North Carolina at Chapel Hill.

International patent application, WO 2005/115482, entitled, “Methods for Identifying Chemical Modulators of Ras Superfamily GTPase Activity,” filed on Dec. 8, 2005.

No publication have arisen from this funding.

Conclusions

Dose-responsive curves must be carried out on the identified hits to establish preliminary IC50s and associated stoichiometries of binding. To rule out experimental artifacts associated with the screening procedure, the identified hits will be confirmed in secondary assays. Two complementary assays will be used. First, the compounds will be tested using a conventional, filtration-based exchange assay measuring the rate of release of [^{32}P]-GTP-γS bound to Ras and catalyzed by Sos1. This assay is designed to flag compounds that have unanticipated fluorescent properties that might confound the original assay to produce false positives. The second assay will entail the use of standard gene reporter assays responsive to activated Ras in cultured HEK 293T cells. This assay will provide a preliminary assessment of cellular permeability of the identified hits and their associated capacity to inhibit Ras activation within a cellular context and without potential toxicity.
A more long-term goal of this project will encompass QSAR of selected compounds that have been filtered through the secondary screening procedures described above. In this regard, we are particularly fortunate to participate in a National Center for Drug Discovery Group (P.I. Said Sebti, Moffitt Cancer Center) with extensive synthetic chemistry expertise and a set of scientific goals commensurate with the development of inhibitors of Ras activation.

References

Gottfried, ON, Viskochil, DH, Fults, DW and Couldwell, WT (2006). "Molecular, genetic, and cellular pathogenesis of neurofibromas and surgical implications." *Neurosurgery* **58**(1): 1-16; discussion 1-16.

Kimple, RJ, Jones, MB, Shutes, A, Yerxa, BR, Siderovski, DP and Willard, FS (2003). "Established and emerging fluorescence-based assays for G-protein function: heterotrimeric G-protein alpha subunits and regulator of G-protein signaling (RGS) proteins." *Comb Chem High Throughput Screen* **6**(4): 399-407.

Appendices

None