Children's Hospital of Pittsburgh

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The Children's Hospital of Pittsburgh received $1,039,905 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

New Reporters of Protein Conformation and Compartment Composition – The purpose of this project is to develop new methods for the assessment of protein location and compartment composition that are necessary to determine the mechanism of human diseases and to develop therapeutic options for their treatment. These methods will be developed initially for the common genetic disease, cystic fibrosis (CF). These expressible reporters permit the detection of expressed proteins at exceptional signal-to-noise. Expressed tags that recognize a variety of fluorogens will permit also the determination of compartment composition, permitting functional anomalies attributed to CF to be assessed in primary airway cell cultures. These methods will be valuable for mechanistic evaluation and drug discovery opportunities for CF and a variety of other human diseases that affect cellular protein handling or localization.

Duration of Project

1/1/2010 - 12/31/2013

Project Overview

New reporters of protein conformation and compartment composition are needed for a mechanistic understanding of human diseases and for the discovery of therapeutics. The development of fluorogenic probes and genetically encoded fluorogen-activated protein (FAP) tags, whose interaction generates intense fluorescence signals with minimal background will permit evaluation of membrane trafficking mechanisms, interactions between proteins, and evaluation of the compositional properties of near-membrane compartments. Our goal is to develop and optimize this approach for the low abundance integral membrane protein, CFTR. Mutations in the CFTR gene cause cystic fibrosis, a common, life-limiting genetic disease. The gene encodes an anion channel that regulates the volume and composition of the thin film of airway surface liquid (ASL) in the lungs. The most common CFTR mutant omits phenylalanine at position 508 (ΔF508), inducing protein misfolding and ER associated degradation (ERAD). Experimental conditions can rescue a small fraction of ΔF508 CFTR to the plasma membrane

(PM) where it is partly functional, but mutant protein rescued in this manner remains unstable and is rapidly cleared by peripheral quality control mechanisms. To date, tools have not been available to quantify CFTR density at the cell surface, or to define protein folding in cellular compartments. The development of these tools is vital to our understanding of the mechanisms that retain and degrade CFTR at the ER and PM, and to provide methods for implementing high throughput/high content screening for therapeutics. Furthermore, a key factor in CF lung disease is the buildup of thick mucins that fail to hydrate into appropriate mucin-gel networks due to the acidic ASL in CF airways. Therefore, defining the microenvironment pH, in conjunction with assessment of CFTR delivery and residence time at the PM, is also an issue of critical significance in assessing the efficacy of therapeutics. This proposal will build truly novel fluorescent probes that address each of these fundamental questions, by defining a) protein trafficking to the cell surface, b) protein interactions and colocalization, c) local calcium concentrations, and d) pH within the cell compartments and at the plasma membrane. These new, genetically encoded imaging probes will be of utility for a wide array of other relevant scientific questions in biomedicine beyond the focus area of cystic fibrosis in this proposal.

Specific Aims:

1. Develop pH-sensitive extracellular FAP-tagged proteins as quantitative pH sensors of the internal compartments accessed by mutant and WT CFTRs following their endocytosis. These probes will permit evaluation of the post-endocytic recycling vs. degradation pathways as a function of CFTR disease mutation, to account for the short half-life of rescued ΔF508 at the cell surface. A screen based on these reagents would identify small molecules that improve mutant protein retention at the apical membrane.
2. Develop FAP reporters with differential fluorogen sensitivity, and Tie-Dye fluorogens of different molecular lengths, to probe protein-protein interactions at the cell surface and within cellular compartments. The interaction of CFTR with molecular chaperones, and with an alternative apical anion channel, SLC26A9, will be used as sensors of proper CFTR folding. A dual FAP-CFTR fusion will be constructed to evaluate the potential of bivalent Tie-Dye fluorogens to directly sense the folded state of CFTR.
3. Develop CFTR-fused and apical membrane localized FAP-reporters of extracellular proton and calcium concentration to monitor compositional changes at the apical membrane microenvironment of CF and non-CF HBE during mucin secretion to identify the mechanism of mucin hydration and its impairment in CF.

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Expected Research Outcomes and Benefits

This project will provide new probes of protein localization and sensors of the properties of localized compartments to examine physiological functions at the level of specific proteins. These tools will provide quantitative probes for protein expression, conformation and compartment composition in a broad range of biological applications. They are needed to provide information of basic disease mechanisms and they will also provide assays that can be used for the development of drug screens and the evaluation of new therapeutics. Among these applications are the screening of small molecules for their ability to promote the plasma membrane trafficking of the protein that is defective in the genetic disease, cystic fibrosis. Success in this endeavor will set the stage for the use of this technology in other diseases of protein folding, the list of which grows each year and includes a variety of neurodegenerative diseases. These fluorogenic biosensors will be produced in cell permeant and cell-impermeant forms, which will permit the assessment of cell surface and total protein in the same experiment on the same cells. CF has served as a paradigm for a variety of human diseases of protein conformation that impair folding or result in protein mis-localization or accumulation or degradation. This new technology therefore has the potential to have a major impact on human health.

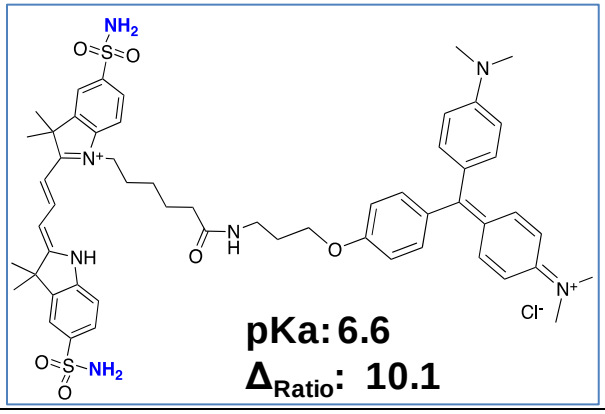
Summary of Research Completed

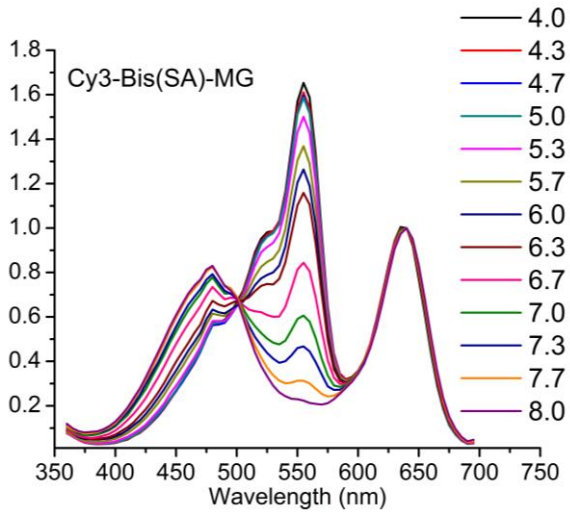
Development of a FAP-based pH sensor: Traditionally, pH sensitive dyes have been solution phase indicators such as SNARF or fluorescein. SNARF is a ratiometric probe generally delivered as an acetoxymethylester to cells. It has the advantage that it will accumulate at high concentrations within the cell. However, it is not possible to measure extracellular pH with the dye, or to specifically direct the molecule to a compartment or protein target. Another option is fluorescein, which quenches non-ratiometrically at low pH. Thus, fluorescein will report extracellular pH, but it is diffusible and can only give a non-reversible measure of change in pH. Its photo-instability often leads to misinterpretation of data. The probe that our collaborators at Carnegie Mellon University have developed is an intra-molecular FRET based reporter which uses the FAP-fluorogen technology to localize a tandem sensor dye (TSD) to a target protein, in this case a physical link to CFTR or to a GPI anchor for apical targeting. The tandem dye component, **Fig. 1, left**, consists of a cell impermeant fluorogen (malachite green--MG) linked to a pH-sensitive variant of a Cy3 dye. A series of FRET-based reporters have now been constructed with different pKa values (6.6, 7.1, and 7.7), covering the range of pH expected in the ASL at the airway surface. This dye has energy transfer efficiency that is highly pH dependent, and is detected as an excitation ratio between the pH dependent channel (561 nm) and the pH independent channel (640 nm); whereas normal physiological pH (7.5) results primarily in excitation at 640 nm, low pH results in activation of the Cy3 donor dye and increased excitation at 561 nm (**Fig. 1, right**). These genetically targeted probes will allow faithful tracking of localized pH changes encountered at the apical surface (GPI anchor) or in the vicinity of the CFTR molecule. As the probe we are using is genetically encoded, but the report of location and pH is based upon a dye molecule binding to the CFTR FAP, we can image its delivery to the cell surface, and assess the pH and pH changes in this compartment under basal and stimulated conditions.

Figure 2 provides a proof-of-concept study using the construct described above. The schematic (**Fig. 2A**) shows the construct as a fusion between FAP and glycophosphatidylinositol (GPI-FAP, brown), which binds to the cell-impermeant fluorogen described above (TSD, gray) and results in fluorogen activation (red). GPI-FAP accumulates at the plasma membrane, where it binds to the cell impermeant TSD, which is quenched when unbound. A sensor pH dye (circle) attached to the fluorogen is quenched in solution, with a secondary excitation activated upon interaction with an analyte (in this case H^+, green), producing an excitation ratio dependent on analyte concentration, with emission only from the fluorogen. Bound to a soluble 'FAP', the dye functions as ratiometric probe for the specific analyte (H^+), and the pH-sensitive excitation is shown (**Fig. 2B**). To monitor extracellular pH modulation, the GPI-FAP was expressed at the membrane in Clone C cells, which express the proton V-ATPase. The cell surface was visualized as a confocal stack from the membrane targeted FAP and the membrane impermeable fluorogen (**Fig. 2C**). TSD has high affinity for the FAP (<1 nM), and displays analyte sensitivity near the donor-only titration values (pKa 7.1 in this case). MG: 640 nm/680 nm, FRET: 561 nm/680 nm. One plane of a confocal stack was selected to measure the emission ratio represented by a pseudocolor, "red" representing more acidic. Sequential images in that plane were obtained. The first image on the second row (framed by a red line) marks the time point closest to the addition to the bath of a cell-permeant V-ATPase activator cAMP analog. Similar confocal sectioning will be used to focus on the apical ASL compartment (see below). The FRET emission ratio measurements (**Fig. 2C**) were used to determine pH_o at the external surface of the plasma membrane (**Fig. 2D**), and as expected, V-ATPase activation lowered pH_o. Cells were maintained at 37ºC throughout the experiment. In the planned studies, we will optimize expression conditions for the constructs and dose-response profiles for the agonists (see below).

Expression in human bronchial epithelia (HBE): As noted above, the approach will be validated using model cell lines, but ultimately, these probes must be targeted to the apical surface of polarized airway epithelial cells. We have had limited time to carry out this work, as this required making an adenovirus vector for expression of an apically targeted FAP in HBE. In this case, we chose to express the GPI-anchored FAP due to the smaller size of its construct. Labeling of polarized, filter grown HBE expressing GPI-FAP is shown in **Fig. 3** at four days after infection. Cells were labeled with the cell-impermeant MG-11p, which we have used in our published studies of CFTR rescue and internalization. The efficiency of expression appears to be ~50%, and the x-y and x-z images clearly show that its expression is apical. There may be a minor amount of MG-11p along the lateral membranes, but the use of confocal sections bracketing the apical border will be used for the ASL measurements.

Fig. 1. pH sensitive tandem sensor dye (TSD). This TSD is based on the cell impermeant fluorogen, malachite green (MG, right), linked to a proton sensitive Cy3 dye.





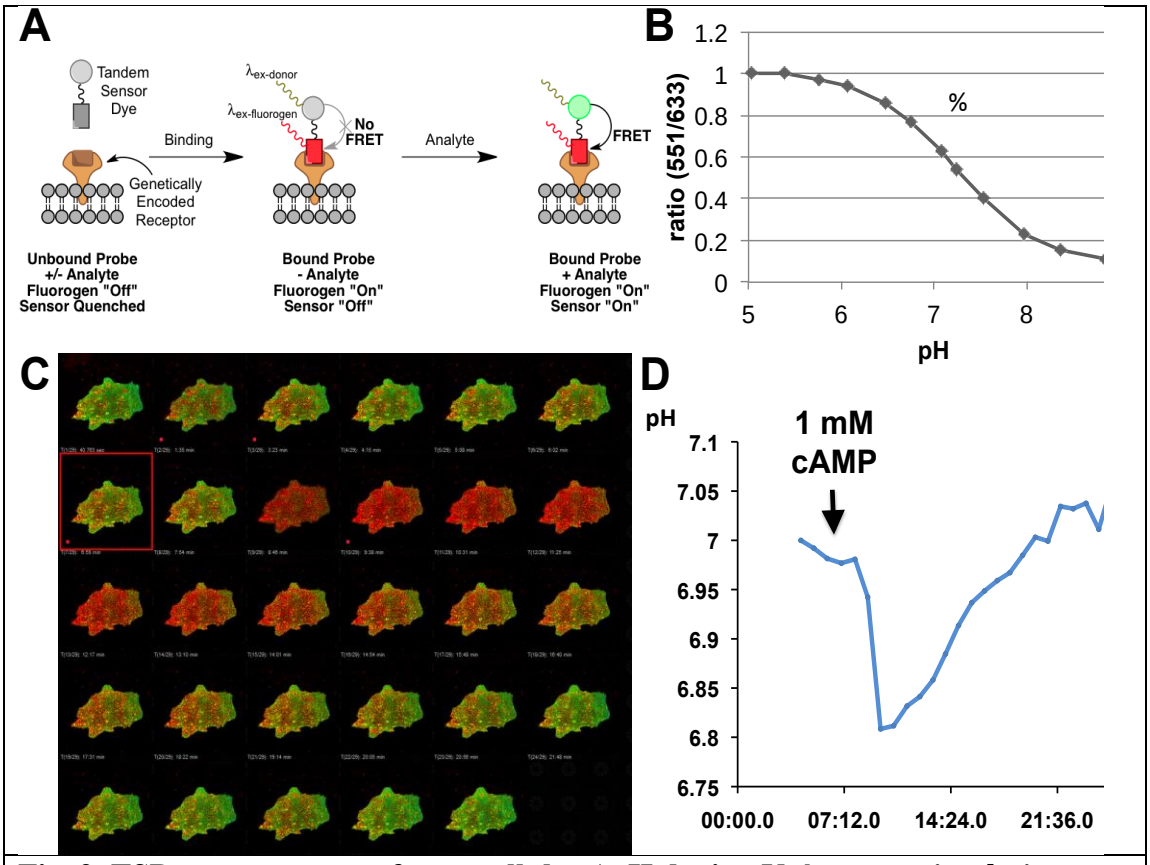


Fig. 2. TSD measurement of extracellular ΔpH during V-ATPase stimulation. See text.

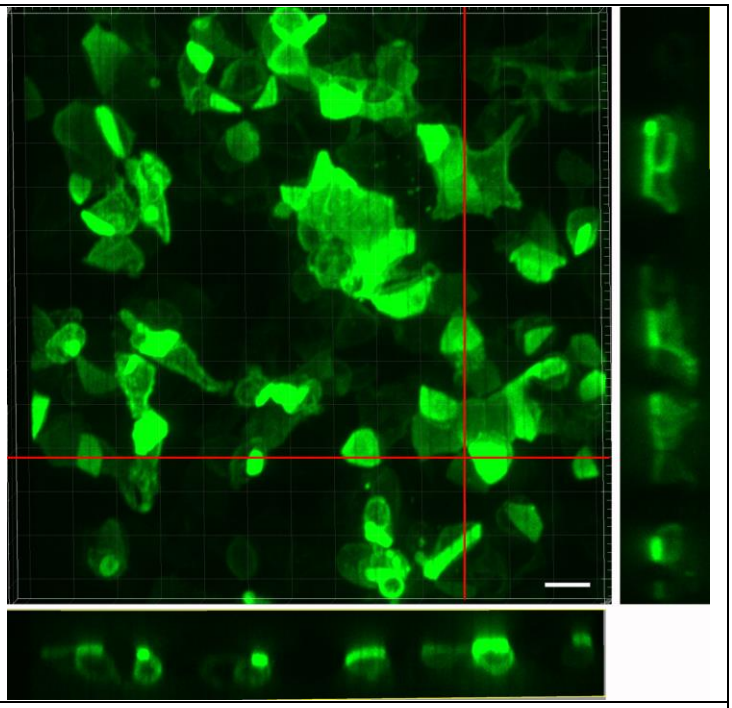


Fig. 3. Fluorogen labeling of GPI-FAP. The membrane anchored FAP was expressed at the apical border of polarized HBE cells.

Research Project 2: Project Title and Purpose

Therapeutic Targeting of Breast Cancer Stem Cells - We have identified a means of inhibiting the differentiation of breast cancer stem cells (CSCs), thus allowing the expansion of this otherwise exceedingly rare and difficult to study population. Characterization of CSCs from 4 different breast cancer cell lines shows them to possess common properties, including a >100-fold increased tumor initiating capacity relative to non-CSCs and resistance to traditional chemotherapeutic drugs. We have identified a number of novel small molecules and siRNAs which selectively inhibit CSC proliferation. We propose to expand these studies to CSCs from primary tumors, to screen for additional small molecule inhibitors, and to better characterize the siRNA-targeted pathways that are necessary for CSC viability. These studies could point the way to new rationally designed strategies for improving breast cancer treatments.

Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Recent work indicates the presence of so-called "cancer stem cells" (CSCs) in many malignant tumors. CSCs are generally rare subsets of tumor cells, which, in contrast to their non-CSC counterparts, are highly efficient at initiating new tumors. Similar to hematopoietic stem cells

(HSCs), CSCs divide slowly but show unlimited self-renewal, which allows them to replenish the CSC pool and provides for an essentially CSC population. Through a type of binary choice which remains incompletely understood, CSCs can also irreversibly differentiate into highly proliferative "transient amplifying cells" (TACs) with a finite replicative capacity. Thus, TACs, either in the proliferative or quiescent state, account for the bulk of most tumors. The CSC hypothesis has spawned the idea that therapy failure is due to inherent CSC chemo-resistance and this has indeed proven to be the cases in several small studies. CSCs are also believed to be more resistant to acidotic and hypoxic conditions found within tumors. Together, these properties of CSCs suggest that their elimination is not only difficult but also essential for complete control over tumor progression to be attained and that novel approaches are needed to eradicate CSCs.

We have recently shown that the Oct3/4 gene promoter region inhibits the differentiation of breast cancer CSC-like cells. As a result, these cells can be expanded indefinitely in vitro. Their inoculation into immuno-compromised mice leads to faster and more efficient tumor growth and the malignant cells recovered from such tumors consist almost exclusively of CSCs. The blocked CSC populations tend to be more resistant to standard chemotherapeutic agents and to hypoxia and acidosis. This has suggested that blocked CSCs can be used to identify novel small molecules or siRNAs that selectively block CSC proliferation and/or survival. We have performed such screens and have identified several small molecules and siRNAs that fulfill this role.

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Expected Research Outcomes and Benefits

One of the major reasons cancers recur is because of failure to eradicate the CSC population. Supporting this idea are studies showing that tumors that recur following chemotherapy commonly contain higher fractions of CSCs than the original tumor from the same individual. Thus, while constituting only a minority cell population, CSCs are believed to be resistant to many standard chemotherapeutic agents and thus to be instrumental in initiating relapse. Moreover, the unlimited proliferative potential of CSCs implies that even extremely small

numbers of surviving cells will be sufficient to initiate tumor re-growth. In contrast, small numbers of TACs would be expected to be lost to attrition due to their limited proliferative abilities. Identifying small molecules and siRNAs that specifically targeted the CSC population might be expected to provide several benefits. First, new drugs that specifically eradicate the CSC population might be identified. The efficacy of such drugs might not have been previously appreciated, as they would not have led to appreciable shrinkage of tumors, whose CSC population is generally small. Second, the combined used of CSC-specific drugs and more traditional agents, which are quite good at attacking TAC populations, might be expected to be synergistic. Finally, siRNAs might identify novel, CSC-specific pathways for which new agents could be developed in much the same way as the recognition of the bcr-abl pathway in chronic myelogenous leukemia led to the development of Gleevec.

Specific Aim 1: The generation of frozen CSCs from other established cells and primary tumors will allow direct comparisons of their different properties and susceptibilities to the CSC-specific agents that we propose to identify in Specific Aims 2 and 3.

Specific Aim 2: Novel agents that selectively target breast CSCs will serve as index compounds for the optimization of therapeutic and pharmacologic properties.

Specific Aim 3: Identifying specific genes whose expression is needed for CSC survival serves to immediately identify the most important pathways upon which CSCs proliferation and expansion depend. This could lead to the design and testing of novel small molecules, specifically and rationally designed to target these pathways.

Summary of Research Completed

Progress for the period July – December 2013:

A. Identification of new celastrol analogs. In our prior Annual Progress Report covering the year ending June 30, 2013, we provided evidence for a new class of compounds with potent inhibitory activity against the c-Myc oncoprotein, a bHLH-ZIP transcription factor that binds to target genes in association with its obligate heterodimeric partner protein Max, which is also a bHLH-ZIP protein. The inhibitory small molecules were all derived from the natural product Celastrol, a quinone methide triterpenoid originally isolated from the Celastraceae family of creeping plants. Many quinone methides have significant biological activities, particularly as anti-inflammatory or anti-tumor agents. Celastrol itself has potent anti-inflammatory properties and it suppresses tumor growth, angiogenesis and metastasis.

Celastrol has a large number of molecular targets including NFk–B, cdc37/Hsp90, ATF2, JNK and numerous chemokines. This promiscuity appears to be the result of a highly reactive quinone methide group (Fig. 1) that allows the molecule to form Michael adducts with free sulfhydryl groups in proteins and small molecules such as glutathione. However, we have demonstrated that this reactive group is not necessary for the inhibition of DNA binding activity by Myc-Max heterodimers (see prior Progress Report). In that report, we showed that several analogs of Celastrol, none of which contained an active quinone methide moiety, could prevent DNA binding by Myc-Max heterodimers. Moreover, three of these compounds promoted a G0/G1

arrest of tumor cell lines, inhibited tumor cell growth in vitro with IC50's of ~8-20 μM and showed modest activity against the growth of Daudi Burkitt lymphoma tumor xenografts in immuno-compromised mice.

B. SBI 0061739: the most potent analog to date. Subsequent to the submission of our prior Progress Report, we have characterized another celastrol analog, SBI 0061739. This compound, like those previously described, lacks the highly reactive quinone methide group (Fig. 1). However, it shows significantly better activity in terms of its ability to disrupt Myc-Max DNA binding and has demonstrated IC50's against HL60 promyelocytic leukemia and Daudi Burkitt's lymphoma cell lines of 100-200 nM. We are currently conducting in vivo testing with the expectation that this compound will prove to be the most potent of all Celastrol analogs thus far identified.

C. A novel mechanism of action for Celastrol analogs. Our previous studies have demonstrated that the inhibitory action of small molecules against DNA binding by Myc-Max heterodimers involved their ability to bind to the monomeric Myc bHLH-ZIP domain, distort its structure and prevent its ability to interact with the cognate bHLH-ZIP domain of Max. However, in the process of characterizing Celastrol and its analogs, we determined that Celastrol (and probably its analogs) operates through a distinctly different mechanism.

To delineate Celastrol's interaction with Myc and Max, our collaborator, Dr. Nick Cosford at The Sanford Burnham Research institute, conducted hetero-nuclear NMR experiments using bacterially expressed, highly purified Myc and Max bHLH-ZIP polypeptides of 83 and 151 residues, respectively. This is an excellent way to document even subtle changes in protein conformation, since the chemical shifts of the observed backbone and side chain H-^{15}N amides are sensitive to perturbations in their chemical environment. Studies can also be conducted under physiologic conditions. Samples were prepared with 100 mM ^{15}N-homogeneously labeled or unlabeled Myc bHLH-ZIP domain and full-length Max(S), respectively, in Tris-HCl buffer with NaCl (150 mM) at pH 7.0. Fig. 2 shows overlays of the resulting spectra. Although only end points of the titrations are shown, changes in the spectra were observed at Celastrol concentrations as low as 5 μM. No effect of Celastrol on Myc alone was detected (panel A). Addition of equimolar ^{14}N-Max to ^{15}N-Myc produced the expected change in α–helical content indicative of PPI (panel B). Adding Celastrol to the complex provoked additional extensive conformational changes in Myc (panel C). Finally, ^{15}N-Max alone was unaffected by Celastrol (panel D). These data suggest that Celastrol inhibits DNA binding by interacting with and altering the structure of the Myc-Max heterodimer without causing its dissociation. It is unlikely that the changes seen in panel C are caused by a non-specific effect such as protein aggregation as the line width of the resonances was unaffected over the entire Celastrol concentration range.

D. A generic assay to quantify the activity of Celastrol and other small molecule Myc inhibitors. We have developed an assay whereby the effects of small molecule Myc inhibitors such as Celastrol or our original compounds 10058-F4 and 10074-G5 can be monitored against generic Myc target or non-Myc target genes in cells. To this end, we stably transfected HeLa cells with a vector encoding a highly labile luciferase protein. The minimal promoter of this vector was engineered to contain tandemly triplicated binding sites for Myc (wt-Myc) or NF-κB. Control

vectors contained either no binding site or a mutant Myc binding site (mut-Myc) (CTCGAG rather than the canonical CACGTG). Relative to the latter control lines whose expression of luciferase was indistinguishable from the background of untransfected cells (not shown), the luciferase activity in cells expressing the wt-Myc and NF-kB vectors were >50 times higher. We then established the conditions under which short-term incubation of these cells with a Myc inhibitor (10074-G5) could be quantified. As seen in Fig. 3, one of our originally described compounds, 10074-G5 showed a dose-dependent inhibition of luciferase in cells expressing the wt-Myc vector, whereas no inhibition of luciferase was observed in cells expressing the NF-κB vector. From these studies, we conclude that 10074-G5 selectively suppresses the expression of genes containing Myc binding sites. We are currently employing this assay against celastrol and its analogs as well as several additional small molecule Myc inhibitors.

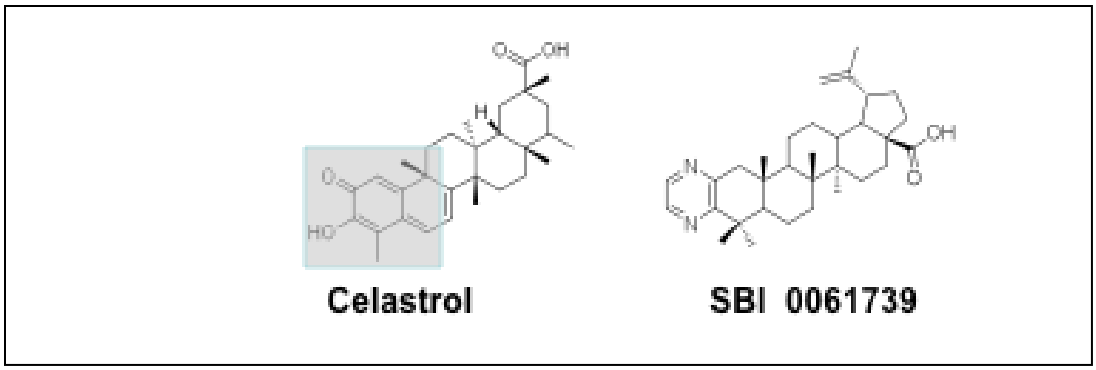

Fig. 1. Structure of Celastrol and the analog SBI 0061739. Note that the quinone methide moiety of Celastrol (gray box) has been altered in the analog

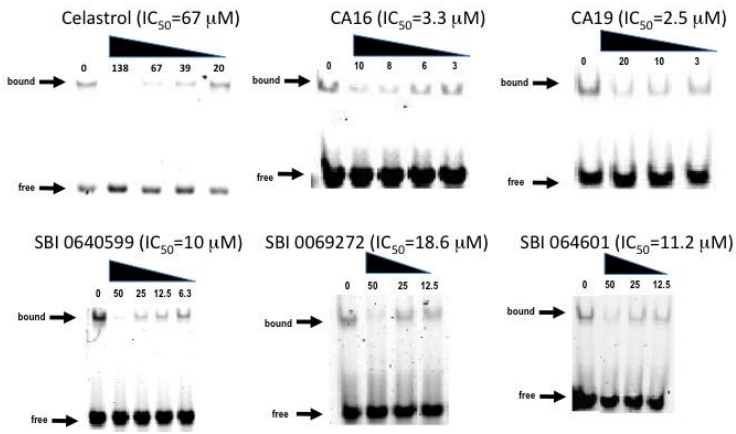

Fig. 1A. EMSA results with Celastrol and its analogs. These studies were performed essentially as described previously (3,9,10) using 30 nM each of bacterially expressed His_6-$Myc_{353-437}$, His_6–$Max(S)_{2-151}$ and a fluorescently-tagged (FAM) double stranded 18-mer oligonucleotide containing a consensus Myc binding site. Control showed that concentrations of compounds up to 100 mM were unable to disrupt homodimer formation by Max(L) (not shown).

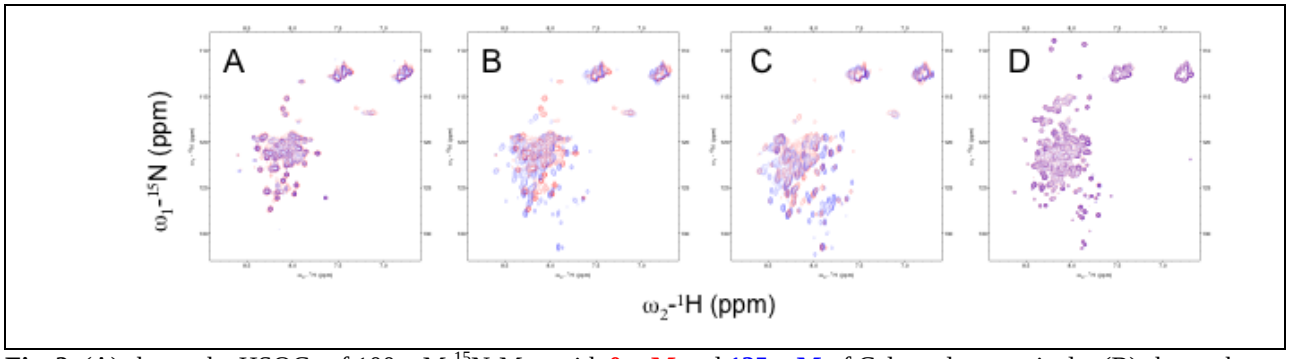

Fig. 2: (A) shows the HSQCs of 100 mM ^{15}N-Myc with **0 mM** and **125 mM** of Celastrol respectively. (B) shows the addition of equimolar (100 mM) amounts of ^{14}N-Max to ^{15}N-c-Myc with the complex spectrum shown in **blue**. (C) shows **0 mM** and **125 mM** of Celastrol on the ^{15}N-Myc/^{14}N-Max complex . (D) shows **0 mM** and **125 mM** of Celastrol on the spectrum of 100 mM ^{15}N-Max alone.

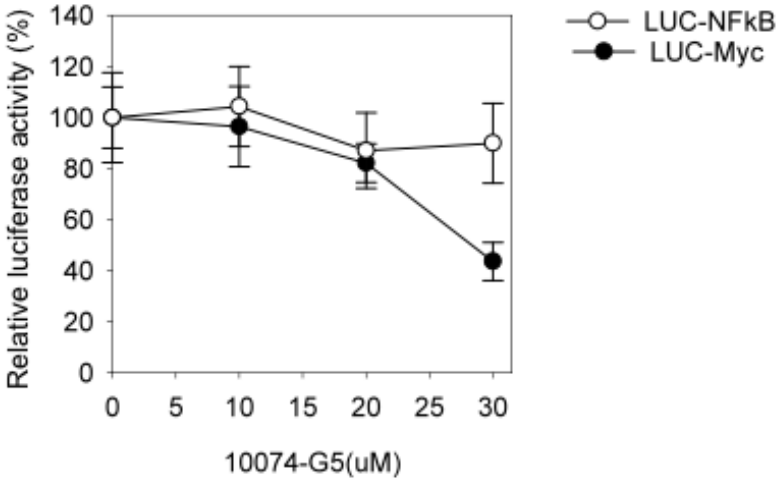

Fig. 3. 10074-G5 specifically inhibits a Myc reporter vector. HeLa cells stably expressing luciferase vectors under the control of a minimal promoter bearing Myc or NF-kB binding sites were exposed to the indicated concentrations of 10074-G5 for 6 hr. Cells were the assayed for luciferase activity. The results depicted represent the mean of triplicate determinations +/- 1 standard error with the value for untreated cells arbitrarily being set to 100%.