Published in final edited form as:

Comb Chem High Throughput Screen. 2008 June ; 11(5): 382–395.

G Protein $\beta\gamma$ Subunits as Targets for Small Molecule Therapeutic Development

Alan V. Smrcka^{*}, David M. Lehmann, and Axel L. Dessal

Departments of Pharmacology and Physiology And Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA

Abstract

G proteins mediate the action of G protein coupled receptors (GPCRs), a major target of current pharmaceuticals and a major target of interest in future drug development. Most pharmaceutical interest has been in the development of selective GPCR agonists and antagonists that activate or inhibit specific GPCRs. Some recent thinking has focused on the idea that some pathologies are the result of the actions of an array of GPCRs suggesting that targeting single receptors may have limited efficacy. Thus, targeting pathways common to multiple GPCRs that control critical pathways involved in disease has potential therapeutic relevance. G protein $\beta\gamma$ subunits released from some GPCRs upon receptor activation regulate a variety of downstream pathways to control various aspects of mammalian physiology. There is evidence from cell-based and animal models that excess $G\beta\gamma$ signaling can be detrimental and blocking $G\beta\gamma$ signaling has salutary effects in a number of pathological models. $G\beta\gamma$ regulates downstream pathways through modulation of enzymes that produce cellular second messengers or through regulation of ion channels by direct protein-protein interactions. Thus, blocking $G\beta\gamma$ functions requires development of small molecule agents that disrupt $G\beta\gamma$ protein interactions with downstream partners. Here we discuss evidence that small molecule targeting $G\beta\gamma$ could be of therapeutic value. The concept of disruption of protein-protein interactions by targeting a “hot spot” on $G\beta\gamma$ is delineated and the biochemical and virtual screening strategies for identification of small molecules that selectively target $G\beta\gamma$ functions are outlined. Evaluation of the effectiveness of virtual screening indicates that computational screening enhanced identification of true $G\beta\gamma$ binding molecules. However, further refinement of the approach could significantly improve the yield of $G\beta\gamma$ binding molecules from this screen that could result in multiple candidate leads for future drug development.

Keywords

G protein $\beta\gamma$ subunits; GRK2ct; computational screening; G protein-coupled receptor; small molecule targeting; protein-protein interactions; G protein signaling

INTRODUCTION

G protein coupled receptors (GPCRs) are a major class of transmembrane receptors responsible for recognition of a large class of diverse ligands. GPCRs in turn regulate a multitude of physiological functions through activation of heterotrimeric G protein α and $\beta\gamma$ subunits that directly bind to target proteins to initiate cellular signal transduction cascades. Nearly 800

GPCRs have been identified in the human genome and include both chemosensory receptors as well as receptors for endogenous ligands. Approximately 380 of the GPCRs in humans are responsible for recognition of endogenous signals but for 150 of these no ligands have been identified [1]. Many GPCRs are important targets for currently marketed drug classes and because of the variety of physiological ligands and participation in multiple human physiologies the GPCRs are major targets for future pharmaceutical development [2]. Since individual ligands can bind to multiple receptor subtypes considerable effort has been placed on identifying selective agonists and antagonists that can activate or inhibit specific GPCR subtypes in specific cell types responsible for specific physiologies and pathologies with limited effects on other systems and therefore fewer side effects. Turning on or off all of the signaling pathways downstream of a particular receptor with specific GPCR agonists or antagonists has been very successful in the treatment of many diseases. An emerging concept, however, is that many pathologies may involve a complex array of receptor signaling pathways where blocking a single receptor completely with a specific high-affinity antagonist may not have the therapeutic efficacy of targeting multiple receptors partially [3]. Another emerging idea is that developing therapeutic agents that block proteins that regulate GPCR function, such as regulators of G protein signaling (RGS proteins), could alter the specificity, potency and efficacy of existing pharmacological agents that target GPCRs [4,5]. The ultimate success of these types of approaches relies on identification of “druggable” targets downstream of the receptor with the requisite specificity so that broadly targeting this molecule will not result in major side effects. Recent success at identification of selective small molecule inhibitors of G protein $\beta\gamma$ subunits [6] suggests another potential approach to modification of signaling pathways downstream of GPCRs that could ultimately lead to novel therapeutics.

HETEROTRIMERIC G PROTEINS AND SIGNALING BY G $\beta\gamma$ SUBUNITS

Heterotrimeric G proteins consisting of multiple isoforms of distinct $G\alpha$, $G\beta$ and $G\gamma$ subunits mediate the actions of a wide variety of cell surface receptors [7–9]. Receptors catalyze exchange of tightly bound GDP for GTP on the $G\alpha$ subunit in a process that requires all three subunits. Binding of GTP results in activation of the G protein and dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ subunits. It is now well understood that the $G\alpha$ and $G\beta\gamma$ subunits both interact with effector molecules, such as phospholipases and ion channels, in a manner that leads to their activation [10]. A variety of *in vitro* studies have shown that when $G\beta\gamma$ subunits are bound to $G\alpha$ -GDP they are incapable of activating downstream effectors [11,12]. Thus, activation and deactivation of $G\beta\gamma$ subunit-mediated signal transduction in cells is thought to rely either on dissociation and reassociation of GTP and GDP-bound α subunits [7], respectively, or GTP dependent conformational alterations in the G protein heterotrimer [13, 14]. Comprehensive reviews of G protein and $G\beta\gamma$ subunit signaling have been published. The goals of this review are to specifically consider the rationale and strategy for small molecule targeting of G protein $\beta\gamma$ subunits. Readers are referred to more comprehensive reviews for a more in depth discussion of G protein $\beta\gamma$ signaling properties [10,15–17].

Multiple combinations of the 5 $G\beta$ and 12 $G\gamma$ subunit isoforms [18] are possible, yet the physiological significance of this diversity is unclear. There are few clear biochemical differences *in vitro* [19,20], but experiments with antisense oligonucleotides [21,22], $G\gamma$ -subunit directed ribozymes [23,24] or genetic deletion of $G\gamma$ -subunits in mice [25,26] suggest very specific roles of $G\beta$ and $G\gamma$ isoforms in receptor signaling in intact cells. Individual $G\beta$ and $G\gamma$ subunit isoforms have unique tissue distributions [27]; although the functional significance of specialized localization of $G\beta$ and $G\gamma$ subunits is not clearly understood. $G\beta\gamma$ subunit isoforms also have differential distributions within the cell. $G\gamma_5$ for example is localized to stress fibers and focal adhesions [28] while $G\gamma_{11}$ can translocate between the plasma membrane and the Golgi [29]. The properties and functions of individual $G\beta\gamma$ subunit

combinations have implications for pharmaceutical development if ligands specific for individual G $\beta\gamma$ subunit combinations could be identified.

PHYSIOLOGICAL SIGNIFICANCE OF G $\beta\gamma$ -DEPENDENT REGULATION OF SIGNAL TRANSDUCTION PATHWAYS

The first physiological mammalian system where G $\beta\gamma$ -dependent signaling was implicated was in direct regulation of inwardly rectifying K_{ACh} channels downstream of muscarinic acetylcholine receptors in atrial cardiac myocytes. Here, G $\beta\gamma$ -mediated activation of this channel modulates membrane potential and heart rate in response to vagal stimulation [10]. Since this initial discovery G $\beta\gamma$ signaling has been shown to be involved in a wide range of cellular physiologies. Genetic deletion of G β or G γ subunits is likely to have pleiotropic effects related to their central role in the G protein cycle. For this reason, physiological roles for G $\beta\gamma$ -dependent regulation of downstream pathways have been explored through knockout of G $\beta\gamma$ target molecules or over-expression of protein based G $\beta\gamma$ inhibitors that block interactions with downstream targets. For example, elimination of G $\beta\gamma$ -responsive phospholipase C by gene-targeting in mouse neutrophils resulted in increased chemotaxis in response to chemotactic peptides and in resistance to viral infection [30]. Genetic deletion of the p110 subunit of G $\beta\gamma$ -regulated PI3K γ in mice resulted in decreased neutrophil migration and a reduction in inflammation [31,32]. In mice lacking G $\beta\gamma$ -regulated PLC β 3, morphine acting at G_i linked opioid receptors produced painkilling effects at lower doses suggesting that G $\beta\gamma$ -dependent PLC β 3 regulation inhibits signaling by the μ -opioid receptor [33].

Significant advances in our understanding of the physiology of G $\beta\gamma$ signaling resulted from development of the C-terminal pleckstrin homology domain of G protein coupled receptor kinase 2 (GRK2ct) as a protein based inhibitor of G $\beta\gamma$. GRK2ct inhibits downstream signaling by G $\beta\gamma$, but at the same time does not appear to interfere with the central role of G $\beta\gamma$ in the G protein cycle [34]. This is based on the observation that expression of GRK2ct inhibits pathways expected to be regulated by G $\beta\gamma$, but does not block pathways known to be regulated by G protein α subunits such as G α_q , G α_s and G α_i [34–36]. Thus, GRK2 can discriminate between G α and G $\beta\gamma$ -mediated pathways which would not be possible if GRK2ct were interfering with general G protein cycling. GRK2ct has been expressed in cell culture and in animals to implicate G $\beta\gamma$ downstream signaling in a variety of pathways, cellular phenotypes and whole animal physiologies. One example of a cellular signaling pathway whose existence has been confirmed in large part because of studies with GRK2ct is regulation of mitogen activated protein kinase (MAP kinase) pathways by G $\beta\gamma$ [35,37]. Activation of multiple G_{i/o} and G_q-coupled receptors, including thrombin, lysophosphatidic acid (LPA), and acetylcholine receptors, results in a mitogenic response in several cell types. MAP kinases are critical components in the growth-promoting pathways regulated by these receptors. Based in part on inhibition of G_i dependent signals by GRK2ct, G $\beta\gamma$ subunits have been shown to activate MAP kinases (although the exact mechanism is unknown) downstream of G_i coupled receptors, suggesting that G $\beta\gamma$ subunits may mediate the growth-promoting effects of many G protein-coupled receptors [38,39]. For example G $\beta\gamma$ -dependent ERK activation promotes growth of vascular smooth muscle cells involved in vascular restenosis [40] and may drive proliferation of prostate cancer cells [41] downstream of receptors stimulated by lysophosphatidic acid and other mitogens.

THERAPEUTIC RATIONALE FOR G PROTEIN $\beta\gamma$ SUBUNITS AS A TARGET

The diverse functionality of G $\beta\gamma$ signaling in cellular physiology suggests that manipulating G $\beta\gamma$ function could have significant therapeutic potential. The therapeutic usefulness of targeting G $\beta\gamma$ signaling has also been investigated extensively through expression of GRK2ct in mice [40,42–45], and to a lesser extent with other G $\beta\gamma$ binding peptides such as QEHA

[46] as well as with genetic deletion of G $\beta\gamma$ -targets in mice. Therapeutic areas involving G $\beta\gamma$ that have support in the literature are summarized in Table 1. Some specific examples are discussed here.

G $\beta\gamma$ and heart failure

A clear indication where GRK2ct has been used to demonstrate the therapeutic potential of targeting G $\beta\gamma$ is cardiac function and failure. A characteristic of heart failure is loss of β -adrenergic receptor (β AR)-dependent cardiac reserve [47,48]. A prominent hypothesis is that the underlying mechanism involves an increase in the activity of GRK2, a kinase that phosphorylates and desensitizes the β AR as well as other GPCRs [49,50]. During progression to heart failure, sustained elevation of catecholamine levels leads to prolonged stimulation of β AR resulting in chronic desensitization of the receptor by GRK2 [49,51,52]. GRK2 activity is controlled by G $\beta\gamma$ which upon GPCR activation recruits GRK2 to the receptor leading to its phosphorylation and desensitization [53]. Expression of GRK2ct blocks this recruitment and enhances β AR function [50]. In a seminal study transgenic cardiac over-expression of GRK2ct in mice increased cardiac performance in response to β AR stimulation [45]. In follow up studies cardiac over-expression of GRK2ct in murine models of heart failure dramatically rescued cardiac function [44] and adenoviral expression of GRK2ct in cardiac myocytes isolated from biopsies of heart failure patients significantly improved contractile function [54]. These and multiple other studies demonstrate the potential value of blocking G $\beta\gamma$ signaling function in improving cardiac function in disease [43,55].

G $\beta\gamma$ and inflammation

Data from gene knockouts of G $\beta\gamma$ effectors in murine models also suggest that targeting G $\beta\gamma$ -effector interactions could be a viable therapeutic strategy. Deletion of PI3K γ in mice inhibits neutrophil migration in response to chemoattractants [31,32] and inhibits inflammatory responses. PI3K γ activity is directly regulated by G $\beta\gamma$ released from G $_i$ proteins activated by chemokine and chemotactic peptide receptors [56,57] and is relatively selectively expressed in monocytic cells. Because of their roles in neutrophil recruitment, chemokine receptors have been the subject of anti-inflammatory pharmaceutical development [58–64]. A potential problem is the overwhelming complexity of these signaling molecules (multiple chemokines, chemokine receptors, and redundancy amongst these) making it difficult to know which specific receptors to target for conditions such as rheumatoid arthritis. Polychemokine [65] or combinations of different chemokine [66] antagonists have been suggested, but there may be chemokines that act as an agonist at one receptor and an antagonist at another [67]. An alternate approach is specific pharmacological targeting of PI3K γ catalytic activity [68]. In this approach blocking PI3K γ would circumvent the problem with chemokine receptor redundancy by blocking a common signaling integrator of chemokines. Potent inhibitors of PI3K γ have been identified and are effective at preventing inflammatory damage in multiple mouse models of rheumatoid arthritis [68]. One key to the long-term therapeutic success of targeting PI3K for inflammation is the ability to identify antagonists selective for PI3K γ relative to other PI3K isoforms [3]. An approach to achieving such selectivity would be to develop inhibitors that block $\beta\gamma$ -regulation of PI3K γ rather than PI3K γ catalytic activity itself [31]. Since other PI3K isoforms are not primarily regulated by G $\beta\gamma$, a high level of specificity for PI3K γ relative to other PI3K isoforms could be achieved with this strategy without having to develop compounds that selectively bind to highly related kinase active sites.

G $\beta\gamma$ and morphine-dependent antinociception

As described earlier, genetic deletion in mice of PLC β 3, a target of G $\beta\gamma$ -dependent activation, results in an increased analgesic potency of morphine. The underlying molecular pathway by which PLC β 3 appears to suppress μ -opioid receptor-dependent signaling is not defined but

may involve feedback inhibition of the receptor itself. Since PLC β 3 is a direct target of G $\beta\gamma$ -dependent regulation, perturbation of this interaction with small molecules would be predicted to increase the analgesic potency of morphine. This approach has been successful in a murine model of antinociception [6] as will be described.

These are just three examples (Table 1) illustrating that inhibition of G $\beta\gamma$ downstream signaling could have therapeutic utility. Identification of small molecule modulators of G $\beta\gamma$ signaling and validation of these molecules in animal models are important steps in realizing this therapeutic goal.

POTENTIAL PROBLEMS WITH TARGETING G $\beta\gamma$ SIGNALING

To consider G $\beta\gamma$ as a viable therapeutic target there are several issues that must be overcome. First, G $\beta\gamma$ plays a central role in the function of all GPCR signaling systems where G $\beta\gamma$ is required for interaction of the G protein heterotrimer with GPCRs. Genetic deletion of the cellular complement of G $\beta\gamma$'s completely disrupts all GPCR signaling [69]. Thus, G $\beta\gamma$ would have to be targeted in such a way that its central role in G protein cycling is not disrupted. Targeting of specific G $\beta\gamma$ protein-protein interactions without ablating general G $\beta\gamma$ function is a possible approach to this. There is considerable empirical evidence that such an approach could be successful based on the previously discussed results with GRK2ct. The structure of the complex of G $\beta\gamma$ with GRK2 has been solved demonstrating that the c-terminus of GRK2 interacts with G $\beta\gamma$ at the same surface of G $\beta\gamma$ as G α subunit switch II [70]. As discussed earlier, despite binding at the G $\alpha/\beta\gamma$ interface, GRK2ct interferes with G $\beta\gamma$ signaling to downstream targets without disrupting GPCR- dependent G protein activation in general [34]. The basis for this selectivity is unclear, but has strong implications for small molecule development. In particular, a strategy that targets the G $\alpha/\beta\gamma$ interface could be successful at blocking downstream G $\beta\gamma$ signaling without globally disrupting G protein signaling.

Another major consideration is that G $\beta\gamma$ is nearly universally expressed so blocking **all** G $\beta\gamma$ functions might have undesired side effects. While G $\beta\gamma$ subunits are universally expressed, G $\beta\gamma$ -target couplings are more restricted. For example, G $\beta\gamma$ -dependent regulation of PI3K γ is relatively restricted to hematopoietic cells including migrating neutrophils and other immune cells [56,57]. If small molecules could be used to relatively selectively interfere with specific G $\beta\gamma$ -effector couplings, the potential for side effects would be reduced. Finally, as has been discussed, multiple G $\beta\gamma$ subtypes have unique tissue distributions. A strategy targeting specific G $\beta\gamma$ subtypes with small molecules could treat G $\beta\gamma$ -related diseases within a particular tissue or organ system with limited undesired effects. The following sections will discuss our understanding of G $\beta\gamma$ protein-protein interactions, and strategies that capitalize on what is known about G protein $\beta\gamma$ subunit biochemistry and structure, to develop small molecules that could overcome many of the aforementioned roadblocks and be useful therapeutically.

STRUCTURAL NATURE OF INTERACTIONS BETWEEN G $\beta\gamma$ SUBUNITS, G α SUBUNITS AND G $\beta\gamma$ TARGETS

To develop a clinically relevant approach that blocks G $\beta\gamma$ subunit function it is essential to understand how G $\beta\gamma$ interacts with its targets at a structural and biochemical level. Three dimensional crystal structures have been solved for G proteins yielding information about the interaction interfaces between G α and G $\beta\gamma$ subunits and between these subunits and their targets [71–74]. The G β subunit belongs to the WD-40 β propeller family of proteins (Fig. 1A). G β has 7 WD40 repeat sequences with the overall motif [X_{11–14}... GH...X_{44–60}... WD] [75] as well as 7 blades comprised of 4 β strands each (labeled a-d for blade 1 in Fig. 1A). Each blade does not correspond directly with the WD40 repeat sequence. For example, the inner a,b and c β strands of blade 1 are derived from the C terminus of 1st WD40 repeat but the outermost

d strand is from the N terminus of the 2nd WD40 repeat. This arrangement is repeated throughout the 7 blades of the β -propeller with the N terminus of repeat 1 forming the outermost strand of blade 7 to complete the propeller (Fig. 1A). The N-terminus of the $G\gamma$ subunit forms a coiled-coil interaction with the N-terminus of $G\beta$ that extends away from the $G\beta$ propeller while the C terminal portion of $G\gamma$ forms an α helix that packs against the $G\beta$ subunit propeller. The $G\alpha$ subunit has extensive interactions with a portion of the top of the $G\alpha$ subunit and the amino terminal α helix of the $G\alpha$ subunit interacts with the side of $G\beta\gamma$ (Fig. 1B). The switch II region of $G\alpha$ (dark blue helix in Fig. 1B) undergoes significant conformational changes upon binding of GTP that likely reduce the affinity of this region for the top of the $G\beta$ subunit surface that may ultimately result in separation of the subunits [8,16].

Mutagenesis to identify protein interaction surfaces on $G\beta\gamma$ subunits

Evidence from a variety of laboratories supports the broad view that different effectors share an interaction surface on $G\beta\gamma$ subunits at a binding site also associated with the $G\alpha$ subunit, but other regions of $G\beta\gamma$ are involved in unique interactions with individual target proteins. To examine the functional role of the $G\alpha$ subunit binding interface of $G\beta\gamma$ subunits in effector activation, a series of alanine mutants were made in the $G\beta$ subunit at amino acids involved in contacts with the $G\alpha$ subunit [76,77]. Many of the mutants were incapable of activating K^+ channels, PLC β , adenylyl cyclase (AC) and other target molecules supporting the idea that a surface obscured by α GDP is involved in regulating many effectors. Interestingly, distinct sets of amino acids at the $G\alpha$ subunit interface of $G\beta$ seemed to be important for activation of different effectors. For example, a series of alanine substitution mutants at this surface differentially affected $G\beta\gamma$ -dependent regulation of PLC β 2, PLC β 3, ACI and II [76,77]. Other studies showed that the sides of the $G\beta$ subunit propeller structure, outside the $G\alpha$ subunit interface and including the N-terminal coiled-coil region, are also involved in effector recognition [78]. In the majority of these experiments only functional regulation of the $G\beta\gamma$ target protein by the mutant $\beta\gamma$ was tested leaving open the possibility that the mutations may have disrupted regulation of the target protein without disrupting binding. Putative effector binding sites have also been identified in yeast $G\beta\gamma$ and $G\gamma$ subunits. These sites map to regions on $G\beta$ and $G\gamma$ subunits that do not correspond to the $G\alpha$ subunit-binding site [79].

An alternate approach to defining a binding site for an effector on $G\beta\gamma$ was based on mapping the sites for crosslinking of a peptide from the $G\beta\gamma$ target, PLC β 2 [80–82]. These studies identified the amino terminus of $G\beta$ subunits as an alternate binding site for PLC β 2, a site similar to an effector binding domain identified in yeast $G\beta\gamma$ subunits [79]. Together, these studies indicate that multiple target recognition by $G\beta\gamma$ subunits involves distinct and overlapping sets of interactions. That each target may have a unique binding mode suggests that molecules could be developed that selectively block $G\beta\gamma$ subunit interactions with distinct groups of effectors.

Structures of $G\beta\gamma$ protein complexes

$G\beta\gamma$ subunits have been co-crystallized with binding partners other than $G\alpha$ subunits providing detailed atomic level information about two representative $G\beta\gamma$ -target interactions. The binding site on $G\beta\gamma$ subunits for phosducin, a molecule that binds to $G\beta\gamma$ subunits in the visual signal transduction system, extensively, but not completely, overlaps the binding site for $G\alpha$ [83]. These data support the idea that effector-binding sites on $G\beta\gamma$ may only partially correspond to the $G\alpha$ subunit-binding site. Similarly, the primary contact amino acids on $G\beta\gamma$ with the C-terminal pleckstrin homology domain of GRK2 are also involved in interactions with $G\alpha$ subunit switch II [70]. However, while many of the same amino acids on $G\beta$ were involved in the interactions with the $G\alpha$ subunit and GRK2ct and phosducin, the mode of interaction was quite different. For example, the switch II helix of $G\alpha$ and the relevant $G\beta$ interacting residues from GRK2 occupy similar a similar surface on $G\beta$ but the structures of bound $G\alpha$ and GRK2

at this site are quite different (Fig. 2A). These data are generally consistent with idea that the $G\alpha$ switch II binding surface on $G\beta\gamma$ interacts with structurally diverse targets through multiple distinct binding interactions.

PEPTIDES AS SELECTIVE MODULATORS OF $G\beta\gamma$ FUNCTION

As an initial approach to development of selective modulators of $G\beta\gamma$ function, G protein $\beta\gamma$ subunits were used as targets to screen random peptide phage display libraries to discover peptides that could bind to different surfaces of $G\beta\gamma$ [84]. Multiple $G\beta\gamma$ binding sequences were identified in this screen that could be grouped into distinct families based on common sequence characteristics. It was predicted that peptides from each family would bind to separate sites on $G\beta\gamma$ subunits corresponding to different protein interaction surfaces on $G\beta\gamma$. Surprisingly, competition experiments indicated that peptides from all of the families bound to a single overlapping surface on $G\beta\gamma$. To account for the observation that multiple peptide sequences bound to a single site on $G\beta\gamma$ subunits and that a single site was targeted in the random peptide screen, it was proposed that the screen targeted a protein-protein interaction “hot spot” on the surface of $G\beta\gamma$ subunits (see below). Nevertheless, these peptides were selective blockers of effector regulation. One peptide, SIRK, blocked $G\beta\gamma$ -dependent activation of PLC β and PI3K γ *in vitro*, but was unable to block $G\beta\gamma$ -mediated inhibition of voltage-gated calcium channels or $G\beta\gamma$ -mediated inhibition of $G\alpha_s$ -stimulated ACI. This was the first indication that reagents could be developed that selectively block interactions between $\beta\gamma$ subunits and particular effectors.

PROTEIN-PROTEIN INTERACTION “HOT SPOTS” AND SMALL MOLECULES

Disruption of $G\beta\gamma$ signaling requires disruption of $G\beta\gamma$ -effector, protein-protein interactions to be successful. High throughput screening efforts targeted at disrupting protein-protein interactions have not generally been successful [85,86]. One possible explanation that has been proposed is that, because protein-protein binding energetics can be distributed over large surface areas compared to ligands, disruption of a small portion of the binding surface with a small molecule may not be sufficient to disrupt protein binding. Additionally protein interaction interfaces are generally flat leading to fewer potential binding interactions to generate high affinity binding sites for small molecules. Nevertheless there are now a number of examples of recent success at disrupting protein-protein interactions with small molecules [87–90]. One key principle that has emerged is that protein-protein binding energetics are often driven by a concentrated core of amino acids at the interface called “hot spots” and that binding of small molecules to “hot spots” can inhibit protein binding interactions. “Hot spots” are defined as subsets of amino acids in crystallographic protein-protein interfaces that, when mutated to alanine, cause a substantial reduction in binding affinity, defining a core of amino acids within an interface responsible for the majority of the protein-protein interaction binding energy [91–93]. These amino acids tend to be clustered at the center of the interface and in some cases have been shown to have plasticity with regard to binding epitopes of variable size and shape allowing recognition of diverse structures with high affinity and specificity [92]. This flexible property of “hot spots” has been implicated in the ability of various receptors to recognize multiple structurally diverse ligands.

Binding sites discovered in screens of random peptide libraries have often been shown to overlap with mutagenically defined “hot spots” suggesting that “hot spots” reflect innate optimal binding surfaces [94,95]. Taken together, the random peptide library screening studies with $G\beta\gamma$ subunits [84], the recognition of multiple structurally diverse targets by $G\beta\gamma$, and the ability of single alanine substitutions at this surface to disrupt target binding interactions are consistent with a “hot spot” model for effector recognition. These characteristics suggest that

Gβγ could have the inherent capacity to bind small molecules at a “hot spot” and that binding at this “hot spot” would successfully disrupt interactions with its binding partners.

IDENTIFICATION AND SELECTIVITY CHARACTERISTICS OF THE Gβγ “HOT SPOT” BINDING SURFACE

The location of the Gβγ “hot spot” was defined in part by solving the X-ray crystal structure of Gβ₁γ₂ bound to a “hot spot” binding peptide, SIGK (SIGKAFKILGYPDYD) [96]. The SIGK contact surface on Gβ₁ is very similar to that occupied by the switch II region of Gα (Fig. (2A, B) and comprised a similar set of amino acids identified by alanine substitution mutagenesis to disrupt Gβγ target interactions. This is somewhat surprising given that this surface had been thought to be important for recognition of most Gβγ targets, yet SIGK and the related peptide SIRK (SIRKALNILGYPDYD), selectively inhibit Gβγ-dependent regulation of specific effectors [84]. On the other hand, this was consistent with mutagenesis data indicating that mutations within the “hot spot” did not affect Gβγ-regulation of ACI [77].

To assess the contribution of Gβ₁ amino acid residues in the SIGK-binding site toward binding of SIGK and other peptides identified in the phage display screen as “hot spot” binders, Gβ₁ residues within 4 Å of the peptide binding site were individually mutated to alanine. None of these mutations had substantial effects on either steady-state heterotrimer formation or heterotrimer dissociation [96]. Nine different peptides obtained from the original phage display screen, with a diverse array of amino acid sequences, were tested for binding to each individual alanine substituted Gβ subunit mutant in a phage ELISA peptide binding assay [97]. Interestingly, each peptide had a unique set of binding requirements within the “hot spot”.

SIGK and SIRK peptides had the surprising property of causing G protein subunit dissociation from preformed heterotrimers leading to activation of G protein βγ subunit signaling in cells [98]. Other peptides that bound to the “hot spot” could compete with binding of Gα subunits, but did not promote subunit dissociation [100]. The differences in peptide effects appeared independent of the peptide binding affinity, but rather were dependent on specific interactions between SIGK or SIRK peptides with the “hot spot” [96,100]. This reinforces the idea that Gβγ functions can be selectively altered by targeting the “hot spot” with peptides that interact in different ways with specific amino acids at this surface.

VIRTUAL SCREENING OF THE NATIONAL CANCER INSTITUTE (NCI) DIVERSITY LIBRARY

The discovery of peptides that bind at the Gα/βγ interface and selectively affect Gβγ functions suggests that if small molecules could be found that bound within the peptide binding site they might also selectively disrupt target interactions. The Gβγ “hot spot” concept suggested that Gβγ might be a good target for small molecules that could successfully disrupt target interactions. Such molecules would be useful to study G protein biology and to validate a new strategy for therapeutics. The overall screening strategy to identify small molecules that bind to the “hot spot” on Gβγ is depicted in Fig. 3. The diversity library of 1990 compounds from the Developmental Therapeutics program at the National Cancer Institute that represents a larger library of 250,251 compounds was computationally screened for binding to the Gβγ “hot spot” using Sybyl/FlexX virtual docking software. For this screen the “hot spot” was defined as the surface area of Gβγ within 6.5 Å from the SIGK peptide as determined from the crystal structure coordinates [96]. FlexX uses a fragment based docking approach to computationally pose individual molecules in multiple orientations in the binding site and selects top poses to be evaluated by the Sybyl/C-score software module [101]. Five scoring functions, D-score, G-score, F-score, Chem-score, and PMF score [102] and two consensus

scores included in the C-score package were used to evaluate the docked poses. These scoring algorithms evaluate the energetics of the docked conformations to predict which molecules would be the “best” binders. No one scoring function is able to accurately capture all of the physicochemical parameters associated with ligand binding [102–104]. D-score and G-score are force field methods; F-score (the one used by FlexX to select the top poses) and Chem-score are empirical scoring functions; PMF-score is a knowledge-based potential; and C- (consensus) and NC- (normalized consensus) Score are equally-weighted averages of the other, five basic scoring functions. These scoring functions have variable performance based on the nature of the target and the ligands being tested. Generally, virtual high throughput screening (vHTS) has been used to identify molecules that bind to small well-defined binding pockets such as enzyme active sites [105,106]. In application of this method to G $\beta\gamma$ docking, the binding surface is relatively large so finding optimal docked poses could represent a challenge for virtual docking programs as will be discussed further below.

SELECTED COMPOUNDS INHIBIT SIGK PEPTIDE BINDING

Eighty five compounds representing the top 1% from each scoring function were selected and tested for “apparent” binding to the G $\beta\gamma$ “hot spot” by determining if they could inhibit binding of a phage displaying SIGK using an ELISA based approach. The assay, based on the method of Scott et. al [84], is performed in the presence of detergent (0.5% Tween) to eliminate non-specific compound aggregation artifacts [107]. Compounds that inhibited SIGK-phage binding to G $\beta\gamma$ by greater than 50% at 100 μ M were then tested at varying concentrations in the ELISA to determine their “apparent” affinity for the G $\beta\gamma$ “hot spot”. Nine candidate compounds that inhibited SIGK binding with IC₅₀'s ranging from 100 nM to 60 μ M were identified. Further analysis of the mechanism of action of these compounds indicated that the majority of these compounds were acting by a competitive binding mechanism, but one compound, selenocystamine (M308) (100 nM IC₅₀ in the competition ELISA), inhibited G $\beta\gamma$ -dependent by a reversible redox-dependent mechanism. Selenocystamine dependent inhibition of peptide binding to G $\beta\gamma$ persisted after extensive washing to remove it, but was recovered after treatment of G $\beta\gamma$ with 5 mM DTT. This was surprising since G $\beta\gamma$ is not thought to be sensitive to redox conditions, but highlighted the need to assess inhibitors for potential redox-dependent mechanisms. Most of the small molecules that bind to G β consist of planar fused ring systems (Fig. 4) that could form the basis for future design of small molecules that bind to this site with higher affinity.

SIMILARITY SEARCHING TO IDENTIFY STRUCTURE ACTIVITY RELATIONSHIPS

One compound, M119, with high “apparent” affinity for G $\beta_1\gamma_2$ (ELISA IC₅₀ = 200 nM) was selected as a lead to define structure-activity requirements (SAR) for this chemical series for binding to G $\beta_1\gamma_2$ (Fig. 5) The NCI 250,251 compound library was searched for compounds similar to M119 using Sybyl/UNITY chemical similarity searching software. This software creates a two dimensional chemical fingerprint based on the presence or absence of particular structural features [108]. Twenty one compounds were identified with similar structures to M119 and tested for relative G $\beta_1\gamma_2$ binding affinities, and key characteristics required for binding were identified. M119 is a xanthene derivative with a cyclohexane carboxylic acid at the 9 position (Fig. 5). Absence of hydroxyl groups at the 4 and 5 positions of the xanthene moiety reduces the apparent affinity for G $\beta_1\gamma_2$ by 1000-fold. On the other hand, multiple substituents at the 9 position retained significant activity.

PERFORMANCE OF HIGH THROUGHPUT DOCKING IN IDENTIFICATION OF G β γ BINDERS

As discussed, it cannot be determined *a priori* which scoring function will be the best for a particular target [103]. After an initial round of screening one scoring function may prove to have the highest performance. Table 2 shows the number of true “hits” for G β γ binding identified with each scoring function in the top 1% of the NCI diversity set library. For the G β γ “hot spot” none of the individual scoring functions out-performed the others for identification of binding molecules. In the case of the G β γ “hot spot” the large surface area contains multiple potential chemical interacting groups. One hypothesis for why none of the scoring functions was better than the others is that the large surface area consists of multiple small molecule binding sites rather than one that is targeted in most screens against enzyme active sites [103,105,106]. Thus, no one scoring function is able to accurately assess docking for all the binding sites. If particular focused binding sites for individual small molecules become defined by structural determination of ligand bound-G β γ or by site directed mutagenesis, these restricted binding sites could be targeted by vHTS and specific scoring functions defined. Alternatively large protein surfaces could be analyzed with programs designed to identify binding pockets on proteins such as PASS [109]. This approach was recently applied as to identify specific sites on β -catenin to target for virtual screening and identification of druglike β 3-catenin inhibitors [88].

To evaluate the utility of computational screening in identifying small molecule binders to G β γ we randomly chose 341 molecules from the NCI diversity set and compared the “hit rate” for the random set to the hit rate for the top 1% of any of the scoring functions that we used. The criteria for a “hit” was a compound that inhibited SIGK binding in the phage ELISA assay by greater than 50% when tested at 100 μ M. A number of compounds whose inhibition was prevented by incubation with DTT were excluded from the analysis. From this data an enrichment factor can be calculated that is the ratio between the probability of finding an active compound in a subset (the top 1%, in our case) and the probability of finding an active compound anywhere in the library. This analysis revealed that many molecules in the library that bound to G β γ at the “hot spot” that were not ranked highly by any of the computational scoring functions. Secondly, the computational screening increased the hit rate with enrichment factors ranging from 1.4 to 4.1 with a scoring function-independent enrichment factor of 2.5.

Two questions arose from these studies. Why were many of the active compounds not ranked at the top by any of the scoring functions, and are there ways to increase the enrichment factors achieved by this method? The answer to these questions might also be related the size of the site being targeted and the consequent failure of FlexX to find the “correct” pose for some of these molecules. As discussed earlier, the two steps in a vHTS experiment are docking to find the molecules position and configuration in three dimensional space followed by evaluation of the chemical and configurational complementarity using scoring functions. Most vHTS experiments dock small molecule libraries to sites that have a size comparable to the ligand size, reducing the complexity of the search process [105]. The surface surrounding the central tunnel of G β is much larger than a medium-size ligand, so the initial docking search by FlexX for the correct orientation and conformation is more complex. If the correctly docked position is not found in the initial docking screen, it cannot be evaluated using a scoring function. FlexX uses the F-score algorithm to identify the best poses, and the top poses selected from this initial docking are then evaluated by other scoring functions. Thus, the screening relies on the accuracy of the F-score algorithm to predict docked poses that can be further evaluated.

SMALL MOLECULES INHIBIT G $\beta\gamma$ -PROTEIN INTERACTIONS *IN VITRO* AND IN CELLS

While the compounds we identified inhibited interactions between G $\beta_1\gamma_2$ and short peptides such as SIGK, the small molecules could have more difficulty disrupting true protein-protein interactions. A flow cytometry based assay was used to directly assess compound effects on fluorescein labeled G α_{i1} binding to immobilized G $\beta_1\gamma_2$ [110]. The overall G α_{i1} - $\beta\gamma$ interaction surface spans 1800 Å² [72,73] and the dissociation constant (K_d) for G α_{i1} binding to G $\beta\gamma$ is approximately 1 nM [110]. M119 blocked G α binding to G $\beta_1\gamma_2$ with an IC₅₀ value of 400 nM. However, unlike SIGK and related peptides that bind to this surface [100], M119 did not promote dissociation of G α_i from G $\beta\gamma$.

While these experiments demonstrate the ability to block interactions between G $\beta\gamma$ subunits and G α subunits, the primary goal is to inhibit interactions between G $\beta\gamma$ and downstream targets that regulate critical signal transduction pathways involved in disease. G $\beta\gamma$ -target interactions can be assessed by functional *in vitro* assays of G $\beta\gamma$ -dependent regulation of target enzyme activities and through analysis of direct binding interactions. In these assays M119 inhibited G $\beta\gamma$ -dependent activation of PLC β_2 , PLC β_3 and PI3K γ and blocked binding of these effectors and GRK2 to G $\beta\gamma$ in direct protein binding assays [6]. UNITY similarity searching based on an M119 chemical fingerprint identified a compound related to M119, M201, but FlexX docking software predicted that compounds M201 and M119 bound to distinct subsurfaces in the “hot spot”. This suggested that the two compounds might have different effects on G $\beta\gamma$ interactions with targets. M119 and M201 both inhibited GRK2 binding to G $\beta_1\gamma_2$ with similar IC₅₀ values of approximately 5 μ M. While M119 attenuated G $\beta_1\gamma_2$ -dependent activation of PLC β_2 , PLC β_3 and PI3K γ , M201 did not affect PLC γ_2 activation by G $\beta_1\gamma_2$ and potentiated G $\beta_1\gamma_2$ -dependent activation of both PLC β_3 and PI3K γ . Thus, these compounds have selective effects on G $\beta\gamma$ -target interactions *in vitro*.

To test the effects of differentially targeting G $\beta\gamma$ on GPCR signaling in intact cells, M119 and M201 were tested for their ability to modulate fMLP receptor-dependent signaling in differentiated HL60 leukocytes. The fMLP receptor couples to G i in these cells and activates PLC β_2 , PI3K γ , and ERK through G $\beta\gamma$ signaling [31,111]. Pre-treatment of differentiated HL60 cells with M119, attenuated fMLP-induced Ca²⁺ increases; pretreatment with M201 had no effect. fMLP-dependent GRK2 translocation to the membrane fraction of HL60 cells on the other hand was substantially inhibited by incubation with either M119 or M201. Thus, M119 and M201 differentially modulate PLC β_2 regulation by G $\beta\gamma$, yet both inhibit GRK2 binding in intact HL60 cells. Together, these data demonstrate that small molecules have the potential to differentially modulate G $\beta\gamma$ interactions with effectors leading to differential modulation of G $\beta\gamma$ -dependent signaling pathways in cells. These are only two of the diverse compounds identified, suggesting potential for multiple modes of G $\beta\gamma$ -dependent target modulation by small molecules.

EFFECTS OF G $\beta\gamma$ -BINDING SMALL MOLECULES ON GENERAL GPCR SIGNALING

As previously discussed, molecules that interfere with G α interactions with G $\beta\gamma$ have the potential to interfere with receptor-G protein coupling because formation of the G $\alpha/\beta\gamma$ complex is required for productive interactions with GPCRs. Despite this, GRK2ct, which binds at the G $\alpha/\beta\gamma$ interface, does not generally interfere with receptor-G protein coupling. To test this for M119, we examined GPCR coupled systems where M119 would not be predicted to have an effect if its mechanism of action were solely to block G $\beta\gamma$ -dependent downstream signaling. In HEK293 cells stably expressing the G $_q$ -linked M3-muscarinic acetylcholine receptor,

carbachol-dependent increases in Ca^{2+} are through $\text{G}\alpha_q$ activation of $\text{PLC}\beta$. M119 had no effect in this system confirming that M119 does not disrupt receptor coupling to $\text{G}\alpha_q$ or $\text{G}\alpha_q$ -dependent Ca^{2+} mobilization [6]. Stimulation of differentiated HL60 cells with fMLP also results in pertussis toxin sensitive activation of various MAP kinases including ERK1 and ERK2, p38 and JNK [112]. Experiments with cell permeable versions of SIGK (mSIGK) indicate that SIGK does not inhibit $\text{G}\beta\gamma$ -dependent ERK activation [98,113]. Since M119 targets the SIGK binding site and SIGK does not inhibit ERK activation, M119 would not be predicted to inhibit ERK activation. Treatment of HL60 cells with M119, M201 or other related compounds did not block fMLP-induced activation of ERK1 and ERK2 demonstrating that despite inhibiting fMLP-stimulated Ca^{2+} signals, M119 does not block fMLP-dependent G protein activation [6]. Overall, these data demonstrate that M119, like GRK2ct, blocks $\text{G}\beta\gamma$ downstream signaling without disrupting general GPCR-G protein cycling.

IN VIVO EVALUATION OF SMALL MOLECULES

As discussed, targeting $\text{G}\beta\gamma$ may have significant therapeutic potential in several areas. An exciting outcome of the small molecule identification is the ability to test these reagents in whole animal models of physiology and disease. One area of interest is opioid-dependent antinociception based on studies of mice with genetic deletion of the $\beta\gamma$ -regulated enzyme, $\text{PLC}\beta 3$. Compared to wild type animals, $\text{PLC}\beta 3^{-/-}$ mice are 10-fold more sensitive to the antinociceptive effects of the μ -opioid agonist morphine [33]. This suggests that $\text{PLC}\beta 3$ is involved in a feedback inhibition loop that acts at the receptor or some other unknown site that inhibits antinociception (Fig. 6). Since M119 blocks $\text{G}\beta\gamma$ -dependent activation of $\text{PLC}\beta 3$ it was hypothesized that coadministration of M119 with morphine might also increase morphine-dependent antinociception. Co-administration of M119 with morphine intracerebroventricularly (i.c.v) in mice resulted in an 11-fold increase in the analgesic potency of morphine in a 55°C tail flick test but had no effect on morphine-dependent antinociception in $\text{PLC}\beta 3^{-/-}$ mice [6]. These data indicate that this strategy of inhibition of $\text{G}\beta\gamma$ subunit inhibition with small molecules could have therapeutic utility in pain management. As discussed, a key factor in the successful application of this strategy is the ability to partially disrupt $\text{G}\beta\gamma$ functions while leaving the central role of $\text{G}\beta\gamma$ in GPCR signaling intact. $\text{G}\beta\gamma$ subunits regulate many aspects of signaling critical for the actions of opioid agonists including inhibition of N-type Ca^{2+} channels and activation of inwardly rectifying K^+ channels [114] (Fig. 6). If M119 were globally blocking $\text{G}\beta\gamma$ subunit functions, we expect that morphine-induced antinociception would have been attenuated rather than potentiated with M119 co-administration. Thus, this model is an *in vivo* demonstration of the ability of small molecules to partially disrupt $\text{G}\beta\gamma$ function downstream of signaling from a GPCR and to modify the actions of a drug acting at a GPCR.

MOLECULAR BASIS FOR SMALL MOLECULE TARGETING OF $\text{G}\beta\gamma$

Protein-protein interactions are thought to represent difficult drug targets [85]. In this computational screen of a relatively small library, several small molecules were found that bound to $\text{G}\beta\gamma$ with apparent affinities in the sub to low μM range that disrupt $\text{G}\beta\gamma$ interactions with target proteins [6]. Thus, $\text{G}\beta\gamma$ appears to be a good target for small molecule binding. The “hot spot” has been proposed to have unique binding characteristics for proteins and peptides that make it an optimal protein-protein interaction surface [96]. It is possible that the same characteristics that make this a good protein interaction surface also make it a good surface for small molecule binding. While the static conformations of the binding site are well defined from X-ray crystal structures, the key characteristics of the amino acids that allow for selective yet flexible binding are not entirely obvious from examination of these static structures. Some properties of the binding surface that may be relevant are that it contains a high relative concentration of aromatic residues, with many of the amino acids having a higher than average

solvent exposure. Comparison of the positions of the amino acids between multiple crystal structures of G $\beta\gamma$ bound to various target molecules indicates that amino acids at this surface undergo significant positional alterations to accommodate structurally diverse binding partners [96]. These properties are consistent with properties described for protein-protein interaction “hot spots” as preferential protein binding surfaces and may also be associated with the ability to bind to small molecules. Another possible characteristic is that, unlike many flat protein interaction surfaces, the fold of the β -propeller results in a hole at the center of the protein-protein interaction interface that is not flat and may allow more potential interactions for small molecule binding.

MOLECULAR BASIS FOR SMALL MOLECULE SELECTIVITY

Several mechanistic models for selectivity of small molecules for G $\beta\gamma$ -effector interactions can be proposed, some of which are discussed briefly here: 1) Spatial selectivity model: The premise of this idea is that binding to different subsurfaces of the “hot spot” selectively, sterically interferes with particular G $\beta\gamma$ -target interactions depending on the unique binding mode of that target. As discussed earlier, alanine substitution mutagenesis indicates that individual effectors bind to different sets of amino acids in the “hot spot”. Computational modeling predicts unique binding interactions for many compounds, but these ligand binding models require validation using structural and/or mutagenic analysis to support or refute a spatial selectivity model. 2) Kinetic selectivity model: If different compounds bind to G $\beta\gamma$ with different kinetics, and individual G $\beta\gamma$ binding partners bind with unique kinetics, differential rates of binding and dissociation of compounds may impart selectivity to those compounds. For example, despite binding at the G $\alpha/\beta\gamma$ interface, many of the small molecules we have tested do not disrupt GPCR- dependent G protein activation, which is thought to require G $\alpha/\beta\gamma$ interactions. Small molecules may not interfere with this cycle because a slow on rate limits access to a rapidly cycling G $\alpha/\beta\gamma$ heterotrimer [115]. In cases where free G $\beta\gamma$ accumulates to a significant level, this free G $\beta\gamma$ may no longer be participating in G protein cycling and is accessible to inhibition by slowly binding compounds or other inhibitors. These hypotheses will be best addressed by detailed analysis of the kinetics of small molecule binding compared to on and off rate constants of protein binding partners as well as either structural or mutagenic mapping of small molecule binding sites. 3) Chemical selectivity model: Binding of compounds to a single binding site the G $\beta\gamma$ “hot spot” alters the chemistry by presenting unique chemical substituents at the surface that in turn alters the nature of the interaction with effectors. This model could be tested by examining the selectivity characteristics of a related chemical series that have similar binding kinetics and binding sites.

PROSPECTS FOR DEVELOPMENT OF MORE SELECTIVE G $\beta\gamma$ BINDING MOLECULES

The initially identified group of molecules that bind G $\beta\gamma$ have varying levels of selectivity. Finding more selective compounds could simply result from searching a larger library of molecules. Alternate approaches could potentially result once a full understanding of the mechanism for selectivity is appreciated. For example, if targeting a subsurface of the “hot spot” correlates with specific interrogation of a G $\beta\gamma$ binding partner, then once the subsurface is defined structurally (by X-ray crystallography, NMR or mutagenesis) it could be targeted specifically for screening with computational prediction methods to produce compounds with particular selectivity characteristics. On the other hand, if the basis for selectivity resides in the chemistry, defining the properties required for a particular selectivity could be used to design new selective molecules.

Finally another approach to selectivity that would be very powerful is development of G protein $\beta\gamma$ subunit subtype selective binding molecules. There are multiple potential combinations of

G β and G γ subunits with unique tissue distributions that potentially couple to individual receptor subtypes. If small molecules were found that could target individual subtypes the potential for selective therapeutics in specific cell types would be quite high. G $\beta_{1\gamma_2}$ was targeted for development of current small molecule inhibitors of G $\beta\gamma$ subunits. The binding site for SIGK is entirely located on the G β subunit and is 100% conserved at the amino acid level in all of the four G β subunits that associate with G γ subunits. This may indicate that differential targeting of distinct G β subunits may not be fruitful. On the other hand, there may be subtle conformational differences in this site either between G β subunit subtypes or in G β subunits bound to different G γ subunits that could be exploited with subtle differences in small molecule chemistry. This could be explored in detail with structural analysis of small molecule interactions with G β subunits or by setting up differential screening assays against different G $\beta\gamma$ subunit combinations.

DEVELOPMENT OF THERAPEUTIC AGENTS FROM SMALL MOLECULE G $\beta\gamma$ BINDERS

This work together suggest that G $\beta\gamma$ maybe a viable target for therapeutics. Initial animal model studies with morphine dependent antinociception suggest that morphine pain control could be an important indication for application of this approach. Additional areas where preliminary animal model data indicate some efficacy of this approach are inhibition of inflammation and inhibition of development of heart failure. While the molecules that have been developed may or may not have potential as drugs these data with animal models indicate that this approach targeting G $\beta\gamma$ may ultimately be a successful therapeutic strategy.

GENERAL APPLICABILITY OF VHTS TO DISRUPTION OF PROTEIN-PROTEIN INTERACTIONS

There have been a number of recent successes in development of small molecule inhibitors of protein-protein interactions. In a number of cases these molecules were found using vHTS [116,117]. In one example, for the small GTPase Rac1, a binding pocket was identified in a co-crystal structure of Rac1 and its exchange factor Tiam and a combination of UNITY 3D similarity search software and FlexX docking software were used to identify molecules that selectively inhibited Rac1 activation *in vitro* and in intact cells [116]. Another example is disruption of Tcf3 with β -catenin. Here, the ability to disrupt the interaction was first validated with small peptides followed by a vHTS screen using FLO_QXP software to identify potent small molecule β -catenin inhibitors [88]. In both cases, one key to the success of the approach was the ability to identify a binding site within the overall protein interaction surface that could accommodate small molecule binding AND that was part a critical energetic “hot spot” for protein-protein interactions. These successes and the recent success with G $\beta\gamma$ subunits indicate that if such sites can be identified at protein-protein interfaces, the vHTS approach could be generally applicable to identification of small molecule inhibitors of many protein-protein interactions opening up a novel approach to therapeutic drug development.

ACKNOWLEDGMENTS

This work was supported by an NIH grant GM-60286 to AVS.

REFERENCES

1. Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA. Proc. Natl. Acad. Sci. U.S.A 2003;100:4903. [PubMed: 12679517]
2. Jacoby E, Bouhelal R, Gerspacher M, Seuwen K. Chem. Med. Chem 2006;1:760.

3. Ruckle T, Schwarz MK, Rommel C. *Nat. Rev. Drug Discov* 2006;5:903. [PubMed: 17080027]
4. Zhong H, Neubig RR. *J. Pharmacol. Exp. Ther* 2001;297:837. [PubMed: 11356902]
5. Neubig RR, Siderovski DP. *Nat. Rev. Drug Discov* 2002;1:187. [PubMed: 12120503]
6. Bonacci TM, Mathews JL, Yuan C, Lehmann DM, Malik S, Wu D, Font JL, Bidlack JM, Smrcka AV. *Science* 2006;312:443. [PubMed: 16627746]
7. Hamm HE. *J. Biol. Chem* 1998;273:669. [PubMed: 9422713]
8. Sprang SR. *Ann. Rev. Biochem* 1997;66:639. [PubMed: 9242920]
9. Gilman AG. *Ann. Rev. Biochem* 1987;56:615. [PubMed: 3113327]
10. Clapham DE, Neer EJ. *Ann. Rev. Pharmacol. Toxicol* 1997;37:167. [PubMed: 9131251]
11. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. *Nature* 1987;325:321. [PubMed: 2433589]
12. Camps M, Hou C, Sidiropoulos D, Stock JB, Jakobs KH, Gierschik P. *Eur. J. Biochem* 1992;206:821. [PubMed: 1606965]
13. Gales C, Van Durm JJJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M. *Nat. Struct. Mol. Biol* 2006;13:778. [PubMed: 16906158]
14. Bunemann M, Frank M, Lohse MJ. *Proc. Natl. Acad. Sci. U.S.A* 2003;100:16077. [PubMed: 14673086]
15. Robishaw JD, Berlot CH. *Curr. Opin. Cell Biol* 2004;16:206. [PubMed: 15196565]
16. Oldham WM, Hamm HE. *Q. Rev. Biophys* 2006;39:117. [PubMed: 16923326]
17. Gautam N, Downes GB, Yan K, Kisselev O. *Cell. Signal* 1998;10:447. [PubMed: 9754712]
18. Hurowitz EH, Melnyk JM, Chen YJ, Kouros-Mehr H, Simon MI, Shizuya H. *DNA Res* 2000;7:111. [PubMed: 10819326]
19. Ueda N, Iniguez-Lluhi JA, Lee E, Smrcka AV, Robishaw JD, Gilman AG. *J. Biol. Chem* 1994;269:4388. [PubMed: 8308009]
20. Wickman KD, Iniguez-Lluhi JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE. *Nature* 1994;368:255. [PubMed: 8145826]
21. Kleuss C, Scherubl H, Heschler J, Schultz G, Wittig B. *Nature* 1992;358:424. [PubMed: 1322501]
22. Kleuss C, Scherubl H, Heschler J, Schultz G, Wittig B. *Science* 1993;259:832. [PubMed: 8094261]
23. Wang Q, Jolly JP, Surmeier JD, Mullah BM, Lidow MS, Bergson CM, Robishaw JD. *J. Biol. Chem* 2001;276:39386. [PubMed: 11500503]
24. Wang Q, Mullah BK, Robishaw JD. *J. Biol. Chem* 1999;274:17365. [PubMed: 10358098]
25. Schwindinger WF, Giger KE, Betz KS, Stauffer AM, Sunderlin EM, Sim-Selley LJ, Selley DE, Bronson SK, Robishaw JD. *Mol. Cell. Biol* 2004;24:7758. [PubMed: 15314181]
26. Schwindinger WF, Betz KS, Giger KE, Sabol A, Bronson SK, Robishaw JD. *J. Biol. Chem* 2003;278:6575. [PubMed: 12488442]
27. Betty M, Harnish SW, Rhodes KJ, Cockett MI. *Neuroscience* 1998;85:475. [PubMed: 9622245]
28. Hansen CA, Schroering AG, Carey DJ, Robishaw JD. *J. Cell Biol* 1994;126:811. [PubMed: 8045942]
29. Azpiazu I, Akgoz M, Kalyanaraman V, Gautam N. *Cell. Signal* 2006;18:1190. [PubMed: 16242307]
30. Jiang H, Kuang Y, Wu Y, Xie W, Simon MI, Wu D. *Proc. Natl. Acad. Sci. U.S.A* 1997;94:7971. [PubMed: 9223297]
31. Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D. *Science* 2000;287:1046. [PubMed: 10669417]
32. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. *Science* 2000;287:1049. [PubMed: 10669418]
33. Xie W, Samoriski GM, McLaughlin JP, Romoser V, Smrcka A, Hinkle PM, Bidlack JM, Gross RA, Jiang H, Wu D. *Proc. Natl. Acad. Sci. U.S.A* 1999;96:10385. [PubMed: 10468617]
34. Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ. *J. Biol. Chem* 1994;269:6193. [PubMed: 8119963]
35. Koch WJ, Hawes BE, Allen LF, Lefkowitz RJ. *Proc. Natl. Acad. Sci. U.S.A* 1994;91:12706. [PubMed: 7809106]
36. Inglese J, Luttrell LM, Iniguez-Lluhi JA, Touhara K, Koch WJ, Lefkowitz RJ. *Proc. Natl. Acad. Sci. U.S.A* 1994;91:3637. [PubMed: 8170960]

37. Crespo P, Xu N, Simonds WF, Gutkind JS. *Nature* 1994;369:418. [PubMed: 8196770]
38. Gutkind JS. *Sci. STKE* 2001;2000:RE1. [PubMed: 11752597]
39. Luttrell LM, van Biesen T, Hawes BE, Koch WJ, Krueger KM, Touhara K, Lefkowitz RJ. *Adv. Second Messenger Phosphoprotein Res* 1997;31:263. [PubMed: 9344257]
40. Iaccarino G, Smithwick LA, Lefkowitz RJ, Koch WJ. *Proc. Natl. Acad. Sci. U.S.A* 1999;96:3945. [PubMed: 10097143]
41. Daaka Y. *Sci STKE* 2004;216:re2. [PubMed: 14734786]
42. Bookout AL, Finney AE, Guo R, Peppel K, Koch WJ, Daaka Y. *J. Biol. Chem* 2003;278:37569. [PubMed: 12869546]
43. Iaccarino G, Koch WJ. *Assay Drug Dev. Technol* 2003;1:347. [PubMed: 15090200]
44. Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J Jr. Lefkowitz RJ, Koch WJ. *Proc. Natl. Acad. Sci. U.S.A* 1998;95:7000. [PubMed: 9618528]
45. Koch WJ, Rockman HA, Samama P, Hamilton R, Bond RA, Milano CA, Lefkowitz RJ. *Science* 1995;268:1350. [PubMed: 7761854]
46. Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, Diamond I. *Cell* 2002;109:733. [PubMed: 12086672]
47. Brodde OE, Michel MC. *Pharmacol. Rev* 1999;51:651. [PubMed: 10581327]
48. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. *N Engl. J. Med* 1982;307:205. [PubMed: 6283349]
49. Hata JA, Koch WJ. *Mol. Interv* 2003;3:264. [PubMed: 14993440]
50. Rockman HA, Koch WJ, Lefkowitz RJ. *Nature* 2002;415:206. [PubMed: 11805844]
51. Sigurdsson A. *Blood Press Suppl* 1995;1:1–45:1. [PubMed: 7599749]
52. Rundqvist B, Elam M, Bergmann-Sverrisdottir Y, Eisenhofer G, Friberg P. *Circulation* 1997;95:169. [PubMed: 8994433]
53. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ. *Science* 1992;257:1264. [PubMed: 1325672]
54. Williams ML, Hata JA, Schroder J, Rampersaud E, Petrofski J, Jakoi A, Milano CA, Koch WJ. *Circulation* 2004;109:1590. [PubMed: 15051637]
55. Li Z, Laugwitz KL, Pinkernell K, Pragst I, Baumgartner C, Hoffmann E, Rosport K, Munch G, Moretti A, Humrich J, Lohse MJ, Ungerer M. *Gene Ther* 2003;10:1354. [PubMed: 12883532]
56. Stephens LR, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AV, Thelen M, Cadwallader K, Tempst P, Hawkins PT. *Cell* 1997;89:105. [PubMed: 9094719]
57. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. *Cell* 1994;77:83. [PubMed: 8156600]
58. Ogata H, Takeya M, Yoshimura T, Takagi K, Takahashi K. *J. Pathol* 1997;182:106. [PubMed: 9227349]
59. Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I. *J. Exp. Med* 1997;186:131. [PubMed: 9207007]
60. Barnes DA, Tse J, Kaufhold M, Owen M, Hesselgesser J, Strieter R, Horuk R, Daniel Perez H. *J. Clin. Invest* 1998;101:2910. [PubMed: 9637726]
61. Plater-Zyberk C, Hoogewerf AJ, Proudfoot AEI, Power CA, Wells TNC. *Immunol. Lett* 1997;57:117. [PubMed: 9232436]
62. Halloran MM, Woods JM, Strieter RM, Szekanecz Z, Volin MV, Hosaka S, Haines GK III, Kunkel SL, Burdick MD, Walz A, Koch AE. *J. Immunol* 1999;162:7492. [PubMed: 10358204]
63. Podolin PL, Bolognese BJ, Foley JJ, Schmidt DB, Buckley PT, Widdowson KL, Jin Q, White JR, Lee JM, Goodman RB, Hagen TR, Kajikawa O, Marshall LA, Hay DWP, Sarau HM. *J. Immunol* 2002;169:6435. [PubMed: 12444152]
64. Yang YF, Mukai T, Gao P, Yamaguchi N, Ono S, Iwaki H, Obika S, Imanishi T, Tsujimura T, Hamaoka T, Fujiwara H. *Eur. J. Immunol* 2002;32:2124. [PubMed: 12209624]
65. Carter PH. *Curr. Opin. Chem. Biol* 2002;6:510. [PubMed: 12133728]
66. al Mughales J, Blyth TH, Hunter JA, Wilkinson PC. *Clin. Exp. Immunol* 1996;106:230. [PubMed: 8918567]

67. Xanthou G, Duchesnes CE, Williams TJ, Pease JE. *Eur. J. Immunol* 2003;33:2241. [PubMed: 12884299]
68. Camps M, Ruckle T, Ji H, Ardisson V, Rintelen F, Shaw J, Ferrandi C, Chabert C, Gillieron C, Francon B, Martin T, Gretener D, Perrin D, Leroy D, Vitte PA, Hirsch E, Wymann MP, Cirillo R, Schwarz MK, Rommel C. *Nat. Med* 2005;11:936. [PubMed: 16127437]
69. Hwang JI, Choi S, Fraser IDC, Chang MS, Simon MI. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:9493. [PubMed: 15983374]
70. Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ, Tesmer JJG. *Science* 2003;300:1256. [PubMed: 12764189]
71. Noel JP, Hamm HE, Sigler PB. *Nature* 1993;366:654. [PubMed: 8259210]
72. Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB. *Nature* 1996;379:311. [PubMed: 8552184]
73. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR. *Cell* 1995;83:1047. [PubMed: 8521505]
74. Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR. *Science* 1997;278:1907. [PubMed: 9417641]
75. Smith TF, Gaitatzes C, Saxena K, Neer EJ. *Trends Biochem. Sci* 1999;24:181. [PubMed: 10322433]
76. Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shektar LR, Rosal R, Weng G, Yang C-S, Iyengar R, Miller R, Jan LY, Lefkowitz RJ, Hamm HE. *Science* 1998;280:1271. [PubMed: 9596582]
77. Li Y, Sternweis PM, Charnecki S, Smith TF, Gilman AG, Neer EJ, Kozasa T. *J. Biol. Chem* 1998;273:16265. [PubMed: 9632686]
78. Panchenko MP, Saxena K, Li Y, Charnecki S, Sternweis PM, Smith TF, Gilman AG, Kozasa T, Neer EJ. *J. Biol. Chem* 1998;273:28298. [PubMed: 9774453]
79. Leberer E, Dignard D, Hougau L, Thomas DY, Whiteway M. *EMBO J* 1992;11:4805. [PubMed: 1464310]
80. Bonacci TM, Ghosh M, Malik S, Smrcka AV. *J. Biol. Chem* 2005;280:10174. [PubMed: 15611108]
81. Yoshikawa DM, Bresciano K, Hatwar M, Smrcka AV. *J. Biol. Chem* 2001;276:11246. [PubMed: 11145956]
82. Sankaran B, Osterhout J, Wu D, Smrcka AV. *J. Biol. Chem* 1998;273:7148. [PubMed: 9507029]
83. Gaudet R, Bohm A, Sigler PB. *Cell* 1996;87:577. [PubMed: 8898209]
84. Scott JK, Huang SF, Gangadhar BP, Samoriski GM, Clapp P, Gross RA, Taussig R, Smrcka AV. *EMBO J* 2001;20:767. [PubMed: 11179221]
85. Arkin MR, Wells JA. *Nat. Rev. Drug Discov* 2004;3:301. [PubMed: 15060526]
86. Whitty A, Kumaravel G. *Nat. Chem. Biol* 2006;2:112. [PubMed: 16484997]
87. Pagliaro L, Felding J, Audouze K, Nielsen SJ, Terry RB, Krog-Jensen C, Butcher S. *Curr. Opin. Chem. Biol* 2004;8:442. [PubMed: 15288255]
88. Trosset JY, Dalvit C, Knapp S, Fasolini M, Veronesi M, Mantegani S, Gianellini LM, Catana C, Sundstrom M, Stouten PF, Moll JK. *Proteins* 2006;64:60. [PubMed: 16568448]
89. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. *Nature* 2005;435:677. [PubMed: 15902208]
90. Berg T. *Angew.Chem Int. Ed Engl* 2003;42:2462. [PubMed: 12800163]
91. Atwell S, Ultsch M, Devos AM, Wells JA. *Science* 1997;278:1125. [PubMed: 9353194]
92. Delano WL, Ultsch MH, de Vos AM, Wells JA. *Science* 2000;287:1279. [PubMed: 10678837]
93. Wells JA, Devos AM. *Ann. Rev. Biochem* 1996;65:609. [PubMed: 8811191]
94. Fairbrother WJ, Christinger HW, Cochran AG, Fuh G, Keenan CJ, Quan C, Shriver SK, Tom JY, Wells JA, Cunningham BC. *Biochemistry* 1998;37:17754. [PubMed: 9922141]
95. Delano WL. *Curr. Opin. Struct. Biol* 2002;12:14. [PubMed: 11839484]
96. Davis T, Bonacci TM, Sprang SR, Smrcka AV. *Biochemistry* 2005;44:10593. [PubMed: 16060668]
97. Smrcka AV, Scott JK. *Meth. Enzymol* 2002;344:557-576:557. [PubMed: 11771410]

98. Goubaeva F, Ghosh M, Malik S, Yang J, Hinkle PM, Griendling KK, Neubig RR, Smrcka AV. J. Biol. Chem 2003;278:19634. [PubMed: 12649269]
99. Ghosh M, Wang H, Kelley GG, Smrcka AV. Meth. Mol. Biol 2004;237:55.
100. Ghosh M, Peterson YK, Lanier SM, Smrcka AV. J. Biol. Chem 2003;273:34747. [PubMed: 12881533]
101. Rarey M, Kramer B, Lengauer T, Klebe G. J. Mol. Biol 1996;261:470. [PubMed: 8780787]
102. Wang R, Lu Y, Wang S. J. Med. Chem 2003;46:2287. [PubMed: 12773034]
103. Bissantz C, Folkers G, Rognan D. J. Med. Chem 2003;43:4759. [PubMed: 11123984]
104. Chen H, Lyne PD, Giordanetto F, Lovell T, Li J. J. Chem. Inform. Model 2006;46:401.
105. Kitchen DB, Decornez H, Furr JR, Bajorath J. Nat. Rev. Drug Discov 2004;3:935. [PubMed: 15520816]
106. Alvarez JC. Curr. Opin. Chem. Biol 2004;8:365. [PubMed: 15288245]
107. McGovern SL, Caselli E, Grigorieff N, Shoichet BK. J. Med. Chem 2002;45:1712. [PubMed: 11931626]
108. Clark, RD. Relative and Absolute Diversity Analysis of Combinatorial Libraries. In: Ghose, AK.; Viswanadhan, VN., editors. Combinatorial Library Design and Evaluation. New York: Marcel Dekker; 2001. p. 337-362.
109. Brady GP Jr, Stouten PF. J. Comput. Aided Mol. Des 2000;14:383. [PubMed: 10815774]
110. Sarvazyan NA, Remmers AE, Neubig RR. J. Biol. Chem 1998;273:7934. [PubMed: 9525890]
111. Neptune ER, Bourne HR. Proc. Natl. Acad. Sci U.S.A 1997;94:14489. [PubMed: 9405640]
112. Rane MJ, Carrithers SL, Arthur JM, Klein JB, McLeish KR. J. Immunol 1997;159:5070. [PubMed: 9366435]
113. Malik S, Ghosh M, Bonacci TM, Tall GG, Smrcka AV. Mol. Pharmacol 2005;68:129. [PubMed: 15802611]
114. Connor M, Christie MJ. Clin. Exp. Pharmacol. Physiol 1999;26:493. [PubMed: 10405772]
115. Ross EM, Wilkie TM. Ann. Rev. Biochem 2000;69:795. [PubMed: 10966476]
116. Zhong S, Macias AT, MacKerell AD Jr. Curr. Top. Med. Chem 2007;7:63. [PubMed: 17266596]
117. Gonzalez-Ruiz D, Gohlke H. Curr. Med. Chem 2006;13:2607. [PubMed: 17017914]
118. Sondek J, Bohm A, Lambright DG, Hamm HE, Sigler PB. Nature 1996;379:369. [PubMed: 8552196]
119. Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, Lefkowitz RJ. Science 1995;268:1350. [PubMed: 7761854]
120. Lymperopoulos A, Rengo G, Funakoshi H, Eckhart AD, Koch WJ. Nat. Med 2007;13:315. [PubMed: 17322894]
121. Eckhart AD, Ozaki T, Tevaearai H, Rockman HA, Koch WJ. Molecular Pharmacology 2002;61:749. [PubMed: 11901213]
122. Yao L, Fan P, Jiang Z, Mailliard WS, Gordon AS, Diamond I. Proc. Natl. Acad. Sci U.S.A 2003;100:14379. [PubMed: 14605213]
123. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan NE, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A. Nature 2001;410:50. [PubMed: 11242036]

Fig. 1.

Structural representations of Gβγ and Gβγ in complex with Gα. (A) Top view of Gβ₁γ₁ subunits (ribbon representation) modeled using Molsoft ICM from coordinates 1TBG [118]. The seven blades of the Gβ₁ subunit β propeller are in orange and green and are numbered according the convention of Wall and Sprang [73]. Lower case yellow letters indicate the 4 β strands that comprise individual blades of the propeller. The N-terminal helix of the β₁ subunit is in red. The first WD40 repeat is shown in orange. The Gγ₂ subunit is in blue. (B) Side view of Gα_{i1}β₁γ₂ from coordinates 1GP2 [73] rendered with Molsoft ICM. Gα_{i1} subunit is shown in ribbon representation with the N-terminal helix in yellow, the Ras-like domain in green and dark blue and the helical domain in orange. The dark blue region of the Ras-like domain

$G\alpha_{i1}$ is the switch II helix. The β subunit propeller domain is in light green and the N-terminal $G\beta$ helical domain is in red. $G\beta W99$ and $G\beta W332$ are shown in space fill (CPK) representation. $G\gamma_2$ is shown in light blue. The subunits are oriented with membrane anchoring points (lipid modifications at N- α and C- γ) and proposed receptor contact sites (C- α and C- γ) at the top of the panel..

Fig. 2.

Interactions between SIGK, Gα_{i1} subunit switch II and GRK2ct with the "hot spot" on Gβ. (A) Close up view of the relative orientations of the Gβγ binding regions of GRK2 (yellow), Gα_{i1} (purple) and SIGK (pink). The top surface of Gβ is shown at the bottom perpendicular to the plane of the panel. SIGK is from pdb 1XHM, GRK is from pdb 1MOW and Gα_{i1} is from 1GP2 and were modeled with Rasmol and rendered with POV-Ray. (B) Top view of SIGK (dark blue ribbon) bound to Gβ₁γ₂ rendered from pdb coordinates 1XHM with Molsoft ICM software. Some key amino acids delimiting the "hot spot" are shown in space fill (red, GβW332; blue, GβL117; yellow GβD228). The Gβ propeller is in light green the Gβ N-terminal helix is in red, and the Gβ subunit is in light blue.

Fig. 3.

Strategy for identification of Gβγ binding molecules. The Gβγ "hotspot" is first interrogated by virtual high throughput screening (vHTS) followed by testing of candidate "hits" in an ELISA based assay for SIGK peptide binding. Compounds that inhibit peptide binding in the ELISA are then tested for blocking of Gβγ-regulated process *in vitro* and in intact cells. Additionally 2D UNITY chemical fingerprinting software is used to evaluate compounds that inhibit peptide binding in the ELISA to identify compounds with similar chemical and structural features. Similar compounds identified with the 2D UNITY software are then tested in the peptide competition ELISA to determine chemical and structural features required for binding to Gβγ and to find higher affinity binding molecules.

Fig. 4.

Structures of some of the identified Gβγ binding molecules identified by vHTS. Concentrations listed are ELISA IC₅₀ values.

Fig. 5. Structures of M119 (NSC 119910) and related molecules identified in the NCI database. M119B (NSC119892), M119K (NSC119893), M119H (NSC119888), M158C (NSC158110), M260 (NSC2608), M119E (NSC119913), M158B (NSC158113). Concentrations listed are ELISA IC₅₀ values. NB = non binder.

Fig. 6.

Schematic model of μ -opioid receptor signaling and feedback inhibition by $\text{G}\beta\gamma$ -dependent regulation of PLC β 3 activity. Arrows indicate $\text{G}\beta\gamma$ can directly activate PLC β 3 and inwardly rectifying potassium channels and inhibit N type Ca^{2+} channels. M119 can block PLC activation by $\text{G}\beta\gamma$, but may not be affecting ion channel regulation. How blocking PLC activation enhances μ -opioid signaling is not known, but could be due to a blocking an effect on the μ -opioid receptor itself or some other target involved in μ -opioid dependent antinociception (represented by the "?").

Table 1**Rationale for Development of Therapeutic Agents that Inhibit G $\beta\gamma$ Functions**

Indication	Evidence for a Role for G $\beta\gamma$
Inflammation	G $\beta\gamma$ -dependent regulation of PI3K- γ plays a critical role in leukocyte migration in response to chemoattractants [31,32,111].
Pain	G $\beta\gamma$ -dependent regulation of PLC β 3 may inhibit opioid signaling. Blockade of this pathway could increase the potency of μ OR agonists [33].
Heart Failure	GRK2ct expression in cardiac myocytes prevents overload hypertrophy [44,119] and inhibits catecholamine release from adrenal chromaffin cells [120].
Hypertension	Vascular GRK2 overexpression causes hypertension [121].
Drug Addiction	GRK2ct and peptide G $\beta\gamma$ inhibitors inhibit synergistic cooperativity of addictive drugs [46,122].
Prostate cancer	GRK2ct inhibits androgen independent growth of prostate cancer cell lines [41,42].
Arterial restenosis	Adenoviral delivery of GRK2ct prevents restenosis in animal models [40].
Cancer Metastasis	Chemokines direct migration of migrating metastatic cancer cells to sites of metastatic tumor formation [123].

Table 2
Performance of Virtual Screening in Identification of Active Compounds

Scoring Function	Actives Subset FlexX	Tested Subset FlexX	Yield Actives FlexX	Actives Random	Tested Random	Yield Random	EF ^a
Any	7	73	9.5%	13	341	3.8%	2.5
Con-Score	2	19	10.5%	13	341	3.8%	2.8
ChemScore	3	19	15.8%	13	341	3.8%	4.1
D-Score	3	19	15.8%	13	341	3.8%	4.1
F-Score	3	19	15.8%	13	341	3.8%	4.1
Gi-Score	1	19	5.2%	13	341	3.8%	1.4
NC-Score	2	19	10.5%	13	341	3.8%	2.8
PMF-Score	1	19	5.2%	13	341	3.8%	1.4

^aEF is the enrichment factor calculated (yield actives FlexX/yield random testing).