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Development of inhibitors of heterotrimeric $G\alpha_i$ subunits

Kathryn M. Appleton^{a,†}, Kevin J. Bigham^{a,†}, Christopher C. Lindsey^a, Starr Hazard^b, Jonel Lirjoni^a, Stuart Parnham^c, Mirko Hennig^c, and Yuri K. Peterson^{a,*}

^aDepartment of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC 29425, United States

^bDepartment of Pharmacology, Medical University of South Carolina, Charleston, SC 29425, United States

^cDepartment of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, United States

Abstract

Heterotrimeric G-proteins are the immediate downstream effectors of G-protein coupled receptors (GPCRs). Endogenous protein guanine nucleotide dissociation inhibitors (GDIs) like AGS3/4 and RGS12/14 function through GPR/Goloco GDI domains. Extensive characterization of GPR domain peptides indicate they function as selective GDIs for $G\alpha_i$ by competing for the GPCR and $G\beta\gamma$ and preventing GDP release. We modified a GPR consensus peptide by testing FGF and TAT leader sequences to make the peptide cell permeable. FGF modification inhibited GDI activity while TAT preserved GDI activity. TAT-GPR suppresses G-protein coupling to the receptor and completely blocked α_2 -adrenoceptor (α_2AR) mediated decreases in cAMP in HEK293 cells at 100 nM. We then sought to discover selective small molecule inhibitors for $G\alpha_i$. Molecular docking was used to identify potential molecules that bind to and stabilize the $G\alpha_i$ -GDP complex by directly interacting with both $G\alpha_i$ and GDP. $G\alpha_i$ -GTP and $G\alpha_q$ -GDP were used as a computational counter screen and $G\alpha_q$ -GDP was used as a biological counter screen. Thirty-seven molecules were tested using nucleotide exchange. STD NMR assays with compound **0990**, a quinazoline derivative, showed direct interaction with $G\alpha_i$. Several compounds showed $G\alpha_i$ specific inhibition and were able to block α_2AR mediated regulation of cAMP. In addition to being a pharmacologic tool, GDI inhibition of $G\alpha$ subunits has the advantage of circumventing the upstream component of GPCR-related signaling in cases of overstimulation by agonists, mutations, polymorphisms, and expression-related defects often seen in disease.

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*Corresponding author at present address: Dept. of Drug Discovery and Biomedical Sciences, College of Pharmacy, Medical University of South Carolina, 280 Calhoun St., QF415, Charleston, SC 29425, United States. Tel: +1 843 792 3691. petersy@musc.edu (Y.K. Peterson).

[†]These authors had equal contribution.

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Keywords

G-protein; NMR; Synthetic chemistry; Drug discovery; Guanine nucleotide dissociation inhibitor; GPCR; Pertussis toxin; Docking; cAMP

1. Introduction

Heterotrimeric G-proteins are a ubiquitously expressed class of proteins that transduce signals from activated G-protein coupled receptors (GPCRs). In its inactive form, the G-protein heterotrimer, composed of $G\alpha$ and $\beta\gamma$ subunits, is found at the plasma membrane with $G\alpha$ in the GDP bound state. When a GPCR is activated by agonist binding, the receptor undergoes a conformational change which facilitates the exchange of GDP for GTP on the $G\alpha$ subunit. Once activated, the heterotrimer $G\alpha$ and $\beta\gamma$ subunits dissociate and signal to their downstream effectors. The G-protein signal is then terminated when GTP, bound to $G\alpha$, is hydrolyzed to GDP and GDP-bound $G\alpha$ re-associates with $G\beta\gamma$.

There are four major subclasses of heterotrimeric alpha sub-units, $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12/13}$. Of these, a large number of vital hormones such as epinephrine, dopamine, acetylcholine, somato-statin, and angiotensin all signal through the $G\alpha_i$ pathway.¹ $G\alpha_i$ is best described as being the inhibitory isoform of $G\alpha$ that suppresses adenylate cyclase activity leading to decreased cAMP accumulation.²⁻⁴ Other effector proteins coupled to $G\alpha_i$ include c-Src, ERK1/2, phospholipase-C (PLC), and monomeric GTPases.⁵⁻¹² In recent years, GPCR signaling cascades have come to light as primary mediators of inflammatory and oncogenic signaling playing critical roles in invasion, metastasis, and uncontrolled growth.^{13,14} Misregulated signaling at the level of the G-protein is also a major component of multiple cancers leading to the potential that $G\alpha_i$ inhibitors could serve a therapeutic niche.^{11,15,16}

The principal laboratory tool for inhibiting the $G\alpha_i$ subunit is pertussis toxin (PTX), an enzyme complex produced by the bacterial pathogen *Bordetella pertussis*. The toxin is taken into the cell via endocytosis where the active enzyme subunit, the S1 A-protomer, ADP-ribosylates the GDP-bound $G\alpha_i$ subunit of heterotrimeric G-proteins.^{17,18} ADP-ribosylation of the alpha subunit locks the entire heterotrimer in its inactive state and prevents activation by agonist-stimulated receptors.¹⁹ PTX blocks the mitogenic effects of GPCR activation by hormones such as epinephrine, sphingosine-1-phosphate(S1P), lysophosphatidic acid (LPA), cytokines, and serotonin.^{15,20-23} As an enzymatic modifier, PTX efficacy is slow requiring overnight incubations leading to compensatory mechanisms. Alternative inhibitors would be useful when acute pharmacological study is required or the monomeric subunit only needs to be inhibited.

An alternative strategy used endogenously by cells to inhibit the $G\alpha$ subunit is to prevent nucleotide exchange. Cellular proteins containing the G-protein regulatory (GPR) or GoLoco motifs bind to $G\alpha_i$ family subunits and function as GDP dissociation inhibitors (GDI) by preventing nucleotide exchange and subunit activation.²⁴⁻²⁶ GPR/Goloco motif-containing proteins in humans include RGS12/14, GPSM1 (AGS3), GPSM2 (LGN), GPSM3 (AGS4 or G18.1b), GPSM4 (PCP2), Rap1GAP, and WAVE1.²⁶⁻³⁰ These proteins

bind to and stabilize the GDP-bound inactive state of $G\alpha_i$, prevent nucleotide exchange, and block heterotrimer oligomerization.^{24,25,31–35} To date, there are no conclusive reports of an endogenous GDI for any of the other heterotrimeric alpha subunit families beyond $G\alpha_i$ which underscores the critical nature of regulating the $G\alpha_i$. GPR/Goloco motif-containing proteins have been implicated clinically in organism development, addiction, and cancer where they are central in the regulation of cell division and differentiation.^{36–38}

The GPR consensus peptide represents the conserved amino acids found among the eight human proteins that contain a GPR motif.²⁵ The 28 amino acid GPR motif contains 12 conserved residues arranged within a minimum 20mer consisting of charged and hydrophobic microdomains.²⁵ The core conserved residues that are required for activity corresponds to: EE-FF-LL—Q—RMDDQR.³⁵ An X-ray crystal structure of the GPR/Goloco domain of RGS14 supports hydrophobic interactions with the amino terminal residues of the peptide with a hydrophobic patch on $G\alpha_i$, and ionic interactions between the carboxy terminal residues of the peptide and $G\alpha_i$. Also of critical importance the conserved arginine in the core of the peptide has a direct interaction with the bound nucleotide. These three points of contact create an elegant and specific mechanism to create a stable four part complex of G-protein, nucleotide, Mg^{2+} , and GDI. Like endogenous GPR domains, the GPR consensus peptide competes for binding of AGS3 to $G\alpha_i$, inhibits GTP binding to $G\alpha_i$, blocks receptor coupling of $G\alpha_i$, and stabilizes the GDP-bound conformation of $G\alpha_i$.^{25,35}

Here we report the TAT-GPR peptide development strategy and *in vitro* effects on cAMP. In addition to being an intriguing probe to study G-protein pharmacology, the TAT-GPR peptide also serves as a baseline and platform to develop small molecule GDIs. The biochemistry of the GPR domain allows us to exploit native mechanisms in the design of synthetic mimetics. We therefore go on to report the strategy, discovery, and validations of small molecules GDIs designed to mimic the GPR peptide interaction with $G\alpha_i$ and GDI biological effects.

2. Experimental section

2.1. GPR Peptides

Peptides were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX) and United Peptide Corp (Herndon, VA), and peptide mass verified by matrix-assisted laser desorption ionization mass spectrometry.

2.2. Cell Culture

GloSensor HEK293 cells (Promega, Inc.) were maintained at 37 °C in a 5% CO_2 atmosphere and maintained in Dulbecco's Modified Eagle Medium (ThermoScientific #SH30243.01) supplemented with 10% fetal bovine serum and 50 μ g/mL hygromycin B was added for selection.

2.3. GTP γ S Exchange Assay

[³⁵S]GTP γ S (1250 Ci/mmol) (21.8 Ci/mmol) were purchased from Dupont/NEN (Boston, MA). GTP γ S binding assays were generally conducted as described.³⁹ $G\alpha_i$ (100 nM) was

preincubated for 20 min at 24 °C in the presence and absence of GPR peptides. Binding assays (duplicate determinations) were initiated by addition of 0.5 μ M GTP γ S (4.0×10^4 dpm/pmol) and incubations (total volume = 50 μ L) continued 30 min at 24 °C. Reactions were terminated by rapid filtration through nitrocellulose filters (S&S BA85) with 4×4 ml washes of stop buffer (50 mM Tris-HCl, 5 mM MgCl₂, 1 mM EDTA, pH 7.4, 4 °C). Radioactivity bound to the filters was determined by liquid scintillation counting. Nonspecific binding was defined by 100 μ M GTP γ S.

2.4. Computer infrastructure

Molecular manipulations and imaging was performed on desktop computers using freeware and MOE.2011 (CCG). Computationally intensive docking was accomplished with the MUSC Computational Biology Resource Center's Cluster. The CBRC cluster consists of sixteen Dell PE1950 dual quad core nodes running RHEL 4.3 and managed by Platform Computing Open Cluster Stack software with LAVA scheduler. Initial in silico screening was performed against the neutral clean lead 280,357 compound subset of the 2009 ZINC database (<http://zinc.docking.org/>).^{40,41}

2.5. Protein targeting

X-ray coordinates came from the PDB 2OM2 file (G α i1-GDP)⁴² and 2RGN (G α q-GDP).⁴³ Both proteins had GDP-Mg²⁺ as well as a peptide from either RGS14 or p63RhoGEF complex respectively. PDB file 1AGR was selected as a transition state analog mimicking the activated state of G α i1-GTP and was used as a negative control to remove compounds that bound to either state in the docking simulations. In order to better mimic the natural state of the G-protein the sulfur from the Xray GSP was replaced with an oxygen. Receptor viewing and editing was done with CHIMERA 1.4. Receptor setup was based on Dock suggested setting. The selected_spheres file was manually edited to further focus the docking procedure. The bound RGS14 peptide and p64RhoGEF as well as any waters were removed prior to docking. Before analysis or simulations, proteins were protonated at pH 7.4 and structures energy minimized with heavy atoms constrained. The positions of GDP and Mg²⁺ were used as positional seeds to set up the initial compound search.

2.6. Screening Docking Simulations

Ligand screening was performed with UCSF DOCK version 6.3.⁴⁴ DOCK was compiled from source with MPICH2.1 that in turn was compiled with INTEL C and FORTRAN compilers (v 10.1). DOCK used default GRID settings, vdw_AMBER_parm99 energy field and 1000 maximum poses per sphere. For initial screening, all ligands were evaluated in rigid mode. The ligand-receptor energy was scored with the unmodified GRID method of DOCK. Both electrostatic and vDW parameters were calculated. Molecules were ranked according to this rigid docking GRID score with 500 maximum poses per sphere. Best scoring compounds were re-docked in flexible mode, using the same parameters as the rigid docking simulations leading to 210 top predicted compounds.

Additional analysis of the high scoring hits and pharmacophore development were accomplished with MOE 2012(CCG). The top 210 compounds were then docked to G α i1-GDP-Mg²⁺, G α i1-GTP-Mg²⁺, and G α q-GDP-Mg²⁺. Docking was performed in screening

mode where the receptor (in this case the trimeric system of G-protein, nucleotide, and magnesium) was held rigid and the ligands were allowed to flex. MOE docking used Amber12:EHT forcefield and Born solvation model. Simulations were focused on the nucleotide by selecting the magnesium ion as the ligand placement strategy. Initial placement calculated 60 poses per molecule using triangle matching placement with London dG scoring, the top 30 poses were then refined using forcefield placement and Affinity dG scoring. The 0990 focused docking simulations used the same structural pbd file and docking as in the DOCK6 simulations. In this case, initial placement calculated 100 poses using triangle matching with London dG scoring. The top 50 poses were then refined using forcefield refinement and Affinity dG scoring.

2.7. Protein expression and purification

Human G α_i 1 was expressed using a plasmid containing the Profinity eXact[®] (BioRad)⁴⁵ construct of G α_i 1 inserted into pPAL7 and stably transfected into *Escherichia coli* BL21 cells. BL21 cells were grown at 28 °C in LB media made using 15.5 g/L of Difco[™] 2 $\times$ YT (ThermoScientific #244020) plus 7.5 g/L NaCl and 50 mg/L ampicillin. G α_i 1 expression was induced with 30 μ M isopropyl-thiogalactose when OD₆₀₀ was ~0.6. Temperature was reduced to 25 °C at induction, and induction was carried out 12-16 h. Cells were harvested at 3200g for 18 min. G α_q was expressed in Hi5 insect cells using G α_q baculovirus (generous gift of Steven Graber WVU) at 5 $\times$ MOI for 48 h.⁴⁶

Cell pellets were lysed using 100 mM NaPO₄, pH 7.2 supplemented with 150 μ M GDP, 5 mM β -mercaptoethanol, Roche Inhibitor Cocktail[®], and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were sonicated and then centrifuged at 100,000g for 30 min. Supernatants were removed and filtered using 0.45 μ m syringe filter. G α_i 1 was then purified according to the Profinity eXact[®] protocol. G α_q was partially purified by first isolating the low speed pellet (10,000g) after and lysis followed by isolation using high (50 k) and low (30 k) molecular weight cut-off filters (Amicon Ultracel UFC805024 and UFC803024).

2.8. Real time nucleotide exchange assays

Real time nucleotide exchange assays were performed in 96-well clear flat bottom plates (Corning 3596). 600 nM purified G α_i 1 or G α_q was incubated with inhibitor compounds in 10 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, pH7.4 (BODIPY Buffer) for 30 min, and 250 nM of the non-hydrolyzable fluorescent BODIPY FL GTP γ S (Invitrogen) was added to initiate nucleotide exchange. Final well volumes were 50 μ L. Test compounds were dissolved in DMSO and final vehicle concentration was 1% v/v. 1 μ M unlabeled GTP γ S was added after 20 min to assess protein viability. Relative fluorescence units (RFU) were measured using a SpectraMax M5 (Molecular Devices) plate reader. Fluorescence was measured at approximately four minute intervals at 475 nm excitation, 525 nm emission, and 515 nm cut-off wavelengths for one hour.

2.9. Synthetic chemistry

Reactions were monitored by analytical thin-layer chromatography on EM-Science hard layer silic gel-60F-254 plates cut into 1 $\times$ 2.5 cm pieces. Visualization was effected by ultraviolet light (254 nm), followed by staining the plate and drying on a hot plate. The stain

was made with 25 grams, of phosphomolybdic acid, 10 grams of cerium sulfate, 60 mL H₂SO₄, and 940 mL H₂O. The potassium permanganate stain was made with 200 mL H₂O, 1.33 grams KMnO₄, 13.33 grams of K₂CO₃, and 4 mL of 5% NaOH. Chromatography was performed by Teledyne ISCO. In reactions where water was not present by solvent, reagent or by-product, the vessels were flame-dried under a slow argon flow. A slight positive pressure of dry argon was maintained via rubber septa seal during the course of the reaction. The argon stream originated from a regulated high pressure argon tank and was used without further drying. All reactions were stirred with a Teflon-coated magnetic stir bar and stir plate. Removal of solvents was typically done using a Buchi rotary evaporator, Model number 011 connected to house vacuum line. The condenser was cooled by a Haake B3 circulator chilled to 0 °C cold thumb. All reaction solvents were purchased as anhydrous with sure seal tops. ¹H NMR spectra were recorded at 400 MHz on a Bruker spectrometer. All chemical shifts were reported from tetramethylsilane with the solvent resonance of CDCl₃ (7.27 ppm), DMSO-*d*₆ (2.5 ppm). Mass spectra were recorded on a Finnegan LCQ Advantage Max. Reverse phase analytical runs were done on an Agilent 1100 HPLC 95:5 H₂O w/0.1% TFA/MeCN w/0.1% TFA -100% MeCN w/0.1% TFA on a phenomenex Luna 3 micron C18 100 angstrom column 20 × 4 mm, with a flow rate of 1.0 mL/minute.

2.10. 0990CL synthesis

To a flame dried flask cooled under argon is added the amine (1.0equiv), the aryl halide (1.1 equiv), and triethylamine (1.5equiv) The mixture is then taken up in 1.4 mL of ethanol (stored over 4 angstrom mol. sieves). The vessel is sealed, and then heated to 120 °C for a period of 4 days which was determined empirically to give the optimum yield. The contents are concentrated and purified via basic alumina using dichloromethane/Methanol (97:3) ¹H NMR (CDCl₃) δ 7.92 (d, 8.28 Hz, 1H), 7.79 (m, 2H), 7.68 (m, 2H), 7.51 (m, 3H), 7.25 (m, 1H), 4.50 (s, 1H), 1.87 (s, 3H), 1.39 (s, 6H). Mass calcd for C₂₁H₂₁N₅, 343.18. Observed, 344.3 (M+1). The final yield for 0990CL synthesis was ~40%. Congener synthesis is provided in the Supplementary methods.

2.11. STD NMR

Samples for STD NMR experiments were prepared with 34 μM protein and 500 μM ligand. STD NMR data were collected at 298 K on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5 mm cryogenically-cooled QCI-inverse probe and using a standard STD pulse sequence^{47,48} with 30 ms 8.3 kHz spin lock to minimize background protein resonances. Solvent suppression was achieved using the excitation sculpting scheme.⁴⁹ Saturation of the protein signals was performed using a train of 10, 20 or 59 selective 56 dB Gaussian pulses of 50 ms duration, totaling a 0.51, 1.02 or 3.01 s saturation time. The on-resonance frequency was set up at -0.5 ppm, and the off-resonance one was applied to -17.16 ppm. STD spectra were acquired with a total of 2048 transients in addition to 8 dummy scans. Longitudinal *T*₁ relaxation times were determined from an inversion recovery experiment processed and analyzed using Topspin 3.1. STD enhancements at *t*_{sat} = 3 s compensated for perturbations from differential ligand proton T₁-relaxation rates were determined following the procedure outlined by Kemper et al.⁵⁰

2.12. Transfections

For the cAMP GloSensor HEK293 cells, 1 μ g of α_2 adrenergic receptor plasmid DNA was added to a final volume of 500 μ l of OptiMEM in tube 'A', and 2.5 μ l of lipofectamine were added to a final volume of 500 μ l of OptiMEM in tube 'B'. Tubes A and B were vortexed briefly and incubated at room temperature for 5 min. Following incubation, tube B was added to tube A, inverted twice to mix, and incubated at room temperature for 40 min. The 1 mL mixture of DNA, lipofectamine, and OptiMEM was added to a 15 mL conical tube containing 1 mL pre-warmed OptiMEM and 3 mL of serum-free MEM. Following medium aspiration and 1 $\times$ PBS wash, the 5 mL mixture was added to each 10 cm plate and incubated overnight.

2.13. cAMP measurements

Cyclic AMP was measured in GloSensor cAMP HEK293 cell line (Promega, Inc.) in 96-well clear bottom plates using a FLIPR^{TETRA}.⁵¹ For PTX treated wells, PTX was added 24 h prior to the assay to a final concentration of 100 ng/mL. The day of the assay (72 h after transfection), cells were pre-equilibrated in GloSensor cAMP reagent and pre-incubated with test compounds for 20 min prior to initiating the assay. Forskolin and/or UK14304 were added simultaneously and relative luminescence units (RLU) were recorded every 2 s for 600 reads.

3. Results

3.1. The permeable GPR peptide

The GPR peptide is a unique and practical way to study the biochemical properties of the GPR motif, $G\alpha_i$, and GPCR function *in vitro* and *in vivo*. However, a major obstacle to observing the GPR peptide effects within a cell is the inherent lack of peptide permeability through cell membranes. In order to facilitate the entry of the GPR peptide into cells, membrane permeable amino acid sequence tags were explored (Fig. 1A). Numerous membrane permeable sequence tags allow passage of usually impermeable proteins and peptides through the cell membrane.⁵² Two chemically distinct sequences were chosen for comparison. A basic peptide sequence derived from the TAT (aa 48-59, NP_057853) (GRKKRRQRRRPP) protein from HIV.^{53,54} TAT fusions (TAT-SIRK) to a $G\beta\gamma$ binding peptide were able to dissociate $G\alpha_i\beta\gamma$ heterotrimers while stimulating ERK, JNK, PLC and Ca^{2+} release.^{32,55} We also explored a hydrophobic signal-sequence based peptide derived from the FGF related oncogene K-FGF (7-21, NP_001998) (AAVALL-PAVLLALLA) found in humans.⁵⁶ The permeable leader sequences were fused to the amino terminus of the consensus GPR peptide to create a synthetic, cell-permeable GPR peptide.

The inhibition of GTP γ S binding to purified G-protein by the modified GPR peptides was then compared to the unmodified GPR peptide (Fig 1B). A major concern was the possibility that modification of the GPR peptide would perturb the secondary structure of the peptide, rendering it less active. Both of the tagged peptides inhibited GTP γ S binding to purified $G\alpha_i$ with slightly lower potency than observed with the unmodified GPR consensus peptide. FGF-GPR peptide modification exhibited a half-log right shift in IC₅₀ for $G\alpha_i$ while the TAT-GPR peptide exhibited an apparent affinity similar to the unmodified GPR peptide.

TAT modification remained more potent than the FGF modification and, therefore, the TAT-GPR peptide was chosen a better candidate for further studies.

We then assessed the intracellular effects of $G\alpha_i$ GDIs on 2nd messenger cAMP levels using the α_2AR (Fig. 2). $G\alpha_i1$ associates with the α_2AR in multiple cellular backgrounds as well as *in vivo*.^{57–59} Note that due to a presumed deficiency of specificity of the GPR motif across $G\alpha_i$ isoforms, it is not specifically known if $G\alpha_i1$ versus $G\alpha_i2$ (or both) is coupling to the α_2AR within our system. α_2AR expression was optimized to prevent G-protein switching to $G\alpha_s$ in presence of PTX, as demonstrated by the absence of enhanced forskolin (Fsk) induced cAMP signal in presence of PTX. Cells were pre-treated with vehicle, PTX (100 ng/mL, 24 h), or TAT-GPR (0.1 μ M, 1.0 μ M and 10.0 μ M, 20 min). After pre-treatment with GPR peptides, cells were treated with 250 nM Fsk and 10 μ M of the α_2AR selective agonist UK14304. Stimulation with UK14304 reduced the Fsk-stimulated cAMP, and this effect was suppressed by PTX. TAT-GPR was very effective at preventing the $G\alpha_i$ mediated inhibition of cAMP stimulated by UK14304, resulting in cAMP levels that were similar to Fsk alone. Also of interest, TAT-GPR but not PTX, was able to raise basal cAMP levels compared to vehicle treatment ($p = 0.009$ between vehicle and GPR basal).

These data demonstrate TAT-GPR can selectively block $G\alpha_i$ regulation of GPCR mediated adenylyl cyclase activity. In the only *in vivo* experiment using GDIs to date, the TAT-GPR peptide replicates the phenotype of chronic cocaine-treatment by inducing altered craving, locomotor behavior, and increased glutamate transmission when TAT-GPR is infused into the prefrontal cortex of rats.^{38,60,61} Although TAT-GPR is very useful as a pharmacologic probe, as a peptide it has several liabilities in development as a potential therapeutic. The GPR consensus peptide has a molecular weight of 3.2 kDa and a pI of 4.1. TAT-GPR is a 40mer with a mass of 4847 Da, a pI of 10.9, and an instability index of 108. We therefore set out to discover small molecule GDIs.

3.2. Identification of small molecule heterotrimeric GDIs

Studies with the GPR peptide and GPR/Goloco domain proteins have taught us that the GDI interaction minimally only needs three points of contact between the GDI and G-protein. Therefore, a small molecule GDI drug only needs to bind the nucleotide and the protein, and each additional interaction is a gain for affinity and selectivity. The initial phase of small molecule identification was performed via computational docking using the X-ray crystal structure of $G\alpha_i1$ -GDP, the ZINC small molecule database of 280,000 molecules, and DOCK6.^{3,40,42,62} The intent was to identify molecules that interacted with both the protein and embedded nucleotide and therefore inhibit nucleotide exchange. This docking study used the inactive conformation crystal structure of $G\alpha_i1$ -GDP (PDB: 2OM2)⁴² and docked small molecules to a region proximal to the nucleotide binding pocket (Fig. 3A and B). The crystal structure of $G\alpha_i$ -GTP (active conformation; PDB: 1AGR) was used as a counter screen to ensure subunit state selectivity of the identified potential inhibitor molecules.⁶³ The crystal structure of $G\alpha_q$ -GDP (PDB: 2RGN)⁴³ was used as another counter screen to filter virtual hits and ensure subunit isoform selectivity. Only 10 compounds out of 280,000 interacted with $G\alpha_q$ -GDP with a predicted binding energy <-1000 kcal. After ranking and filtering, a total of 210 molecules emerged from the flexible docking after passing thresholds

of -1000 kcal and represented a virtual hit rate of $\sim 0.08\%$ (Fig. 3C). We further validated the DOCK6 based simulations using MOE as an alternate simulation methodology. We redocked the top 210 compounds to the top 210 compounds to $G\alpha_i1$ -GDP- Mg^{2+} , $G\alpha_i1$ -GTP- Mg^{2+} , and $G\alpha_q$ -GDP- Mg^{2+} (Fig 3D). The large scatter and high (poor) scores for $G\alpha_i1$ -GTP and $G\alpha_q$ -GDP indicated that DOCK6 and MOE were in agreement to enrich for compounds that reproducibly interacted with the $G\alpha_i1$ -GDP nucleotide site.

The specific $G\alpha_i1$ -GDP hits were rank ordered according to binding energy and grouped into 65 molecularly similar chemical clusters using a Tanimoto similarity coefficient (Tc) of 60%.⁶⁴ Fifteen representative molecules representing one-quarter of the possible clusters were selected to sample diverse chemical space. These compounds were then validated for their ability to regulate $G\alpha_i$ and $G\alpha_q$.

3.3. Validation of small molecule $G\alpha_i$ GDIs

Real time nucleotide exchange assays were performed on small molecules obtained from the docking screen using the non-hydrolyzable fluorescent nucleotide analog BODIPY FL GTP γ S.^{65,66} To improve sensitivity of the assay, we modified the experimental wavelengths by using an excitation of 475 nm and monitored emission at 525 nm with a 515 nm cut-off. Although this modification only left a 10 nm observation window (515 to 525 nm) and decreased the overall signal intensity, we found that it improved the ability to search for low affinity interactions while decreasing potential fluorescence interference.

We tested the 15 compounds in the primary $G\alpha_i1$ and $G\alpha_q$ exchange assay (Table 1). The exchange assay demonstrated a concentration-responsive reduction in fluorescence for several compounds compared to vehicle control reactions (Fig. 4A and B & Fig. S1). Four compounds showed selectivity for $G\alpha_i1$ over $G\alpha_q$. Selectivity was best demonstrated by compounds **0990** and **4630** which demonstrated selectivity for $G\alpha_i$ and resulted in a maximum endpoint fluorescence reduction of $38 \pm 8\%$ and $34 \pm 3\%$, respectively (Fig. 4C & Fig. S1). Compounds **8005**, **8770**, and **4799** were selective for $G\alpha_q$ with **8005** showing a maximum reduction of endpoint fluorescence of $27 \pm 7\%$ respectively (Fig. 4D & Fig. S1). Compounds **2967**, **6715**, and **1026** were not selective and showed inhibition of nucleotide exchange with both $G\alpha_i1$ and $G\alpha_q$ (Table 1). The remainder of the compounds did not affect nucleotide exchange activity. Compounds that showed selectivity for $G\alpha_i1$ and also a reduction in end-point fluorescence greater than 10% were retained for further testing. This threshold criterion was chosen in order to explore greater chemical space and chemotypes in the early phase of screening. We also re-synthesized **0990** to confirm structure and purity for all further experiments (Supplementary methods, in-house synthesized compound is specifically named **0990CL**).

To confirm binding of **0990CL** to $G\alpha_i$ in solution we used saturation transfer difference (STD) NMR. STD NMR is a robust technique allowing direct measurement of ligand binding to target proteins through ligand signals that appear in STD experiments due to saturation transfer from the target protein while in the ligand's bound state.^{47,67} STD NMR confirmed that compound **0990CL** interacted with purified $G\alpha_i1$ in its GDP-bound state which we used in these experiments (Fig. 5). Interestingly, our NMR analysis revealed that compound **0990CL** converted to the tautomer state shown in Figure 5A in aqueous solution

(H₂O/DMSO-*d*₆; 90:10) exhibiting a deprotonated nitrogen N1' and a sp³-hybridized carbon C3'. Compound **8005**, which showed no inhibition of Gα_i nucleotide exchange, was used as a negative control. Figure 5B and D show the aliphatic and aromatic regions of reference spectra of a mixture of **0990CL** and Gα_i1 in the presence of excess GDP. The STD NMR spectra recorded with varying saturation times (*t*_{sat} = 1 and 3 s) revealed several points of contact on **0990CL** when bound to Gα_i1 (Fig. 5B and D). The relative STD enhancements for individual **0990CL** protons reflect the proximity to the binding site of Gα_i1. However, the T₁-relaxation of individual protons and exchange kinetics can further complicate the analysis of the binding epitope.^{50,68} Therefore, we explicitly accounted for differential longitudinal relaxation times of **0990CL** protons ranging from 2.284 for the aromatic H4'' to 0.422s measured for the aliphatic 2'-Me (Fig 5A).⁵⁰ Normalized STD enhancements (setting one H3' proton to 100%) at a saturation time of *t*_{sat} = 3 s are summarized in Figure 5C. The largest STD effects can be observed for the two aliphatic H3' protons followed by the two 2'-Me and the 4'-Me groups. In the aromatic region, more moderate interactions of the compound with Gα_i were also observed for both aromatic ring systems involving protons 2' through 6' and H5, 6, 7 and 8. Inspection of mixtures of Gα_i1 in its GDP-bound state together with **0990CL** and **8005** revealed that all of the signals of the **8005** negative control were absent in the difference spectrum indicating that this compound does not bind Gα_i1 (Fig. S2).

3.4. Structure–activity relationship survey of **0990CL**

After binding confirmation by NMR, compound **0990CL** was selected for further validation. Six **0990CL** congener compounds were subsequently purchased to explore substituents on the aniline and phenyl rings of a quinazoline scaffold (Supp. Table 1). Compound **9585**, with a methyl group at positions 6 and 5' and compound **9586**, with a methyl at positions 6 and 3', both displayed >50% inhibition of nucleotide exchange with Gα_i. Compound **9587**, with a chloride at 5' and a methyl at position 6, displayed only weak inhibition of nucleotide exchange on Gα_i (~16%). These results suggest that hydrophobic interactions on the aniline ring enhance exchange inhibition. Compound **9253**, with a carbonyl at the position 2', a methyl at 4', and methyl groups at positions 4 and 6, proved ineffective at influencing nucleotide exchange. Also ineffective was compound **8358** with methyl groups at the 4 and 4' position. These two results emphasize the need for the phenyl substituent at position 4 for exchange inhibition. Compound **6784**, with a hydroxyl at the 5' and a phenyl and methyl at positions 4 and 6, respectively, also showed no activity towards nucleotide exchange inhibition. These data indicate the requirement for hydrophobic substituents on the aniline ring and the phenyl ring at position 4.

Our data thus far confirmed the quinazoline scaffold as amenable to GDI activity. We went further and synthesized and tested ten additional congeners or fragments based on compound **0990CL** (Supp. Table 1). Compound **2397**, with a chloride at the 3' position and a phenyl at position 4, and compound **2395**, with a phenyl at position 4 both resulted in >30% inhibition of nucleotide exchange on Gα_i. Compounds **2353** and compound **2369** resulted in 23% and 18% inhibition of Gα_i nucleotide exchange, respectively, suggesting that the quinazoline scaffold enhances exchange inhibition. Compound **2410**, with only a single nitrogen on the aniline ring and phenyl at position 4; and **2437**, with methyl groups at

positions 3' and 5' and a phenyl at position 4 were both inactive toward nucleotide exchange. These results suggest that both nitrogens are likely required for compound interaction. Compound **2471**, with carbonyl linker connecting the quinazoline and the aniline ring, was inactive indicating that the phenyl-quinazoline-aniline core structure enhances compound interaction. Compound **2621**, with a methoxy group at position 3' and a phenyl at position 4, was inactive. Other inactive non-quinazoline type compounds included **2355**, with just a portion the nitrogen trio of the quinazoline-aniline structure; and **8910**, exploring this same feature, emphasize the need for the basic phenyl-quinazoline-aniline root structure.

3.5. Molecular docking study of **0990CL**

To further analyze the potential properties of the inhibitor/G-protein interaction, we performed molecular docking simulations on compound **0990CL**. The top pose of interest for **0990CL** interacted with both Arg¹⁷⁸ and Val¹⁹⁹ (Fig. S4). Energy minimization of the system which allowed the G-protein and nucleotide to flex had no effect on the final pose. Other high probability interactions were the central secondary amine of **0990CL** donating a proton to Glu⁴³ or Gln⁷⁹ and H-arene interactions with Gln⁷⁹ or Lys¹⁸⁰. As we had hoped, these interactions mimic much of the biochemistry of the GPR/Goloco interactions with a mix of hydrophobic and ionic interactions. These poses also indicate that the unmodified phenyl is not planar with the rest of the molecule. Also the central secondary amine is a flexible hinge allowing the overall molecule to be convex. These features help to create a molecule with three dimensional topology to present both hydrophobic and proton donating features.

Through our synthetic, informatics, and structural characterization we observed tautomerization of **0990CL**. Specifically, the 3' carbon (Fig. 5A and C and Fig. S4) on the terminal pyrimidine is capable of switching between alternate sp² or sp³ hybridization. Tautomer T1 was appealing as our earlier pose predictions indicated the potential for the Arg¹⁷⁸ guanidinium group donating a hydrogen bond. However, our STD NMR experiments indicated the sp³ tautomer was the primary interacting form in our experiments. To further understand the consequences of tautomerization of **0990CL** for interaction with Gα_i, we tested the two tautomers in molecular simulations (Fig. S5). Using the same GDP site as used in the primary virtual screening, both **0990CL** tautomers were docked to Gα_i. Docking scores for the top 25 poses between the two were nearly identical. Further investigating the poses, we recognized that the poses were flipped with respect to each other and the GDP binding site. Tautomer T1 consistently had the trimethylpyrimidine buried while T2 had the phenyl portion of the quinazoline buried (Fig. S5C). While both tautomers were able to form effective bonds with Val¹⁷⁹ in the simulations, T2 was able to form additional bonds with GDP. Lastly, we took the top poses for each tautomer and calculated the potential energy for the complex and individual components (Fig. S5D). The poses predicted from the docking simulations gave the lowest energies while swapping the tautomers gave poorer energies. Interestingly, T1 in the T2 pose was very unfavorable in terms of energy, indicating T2 in its native pose is the highest affinity interaction possible.

3.6. Effect of GDIs on cellular cAMP

We also assessed the cellular effects of small molecule $G\alpha_i$ GDIs on cAMP levels using the α_2AR as a cellular model (Fig. 6 and Fig. S3). **0990CL** was able to partially restore cAMP levels following UK14304 treatment. Restoration was greatest at the lowest concentration of **0990CL** (100 nM), which was able to restore ~31% of the reduction of cAMP elicited by Fsk and UK14304. At higher concentrations of **0990CL** (10 μ M), cytotoxic effects of the compound began to negatively impact cell viability. The negative control compound **2355** showed no effect upon cAMP levels following treatment with Fsk and UK14304 which demonstrates our inhibitors likely do not interfere with the ability of UK14304 to activate the α_2AR . Also of note, **8770** and **2967** were able to suppress cAMP signaling through α_2AR in a concentration-dependent manner. Compounds **8770** and **2967** demonstrated a lack of toxicity at 10 μ M indicated by measured max efficacy of Fsk in the presence of UK14304. The fact **8770** did not inhibit $G\alpha_i1$ in our nucleotide exchange assay yet shows *in vitro* efficacy highlights the necessity to conduct multiple assays to fully characterize compounds as inhibitors in any drug screening platform.

4. Discussion

Heterotrimeric G-proteins are central regulators of cellular communication, and potent and selective inhibitors can serve multiple purposes ranging from being a pharmacologic probe to experimental therapeutics. As G-proteins are signaling amplifiers, they are an ideal target for therapeutic intervention and diagnosis. Here we presented the rationale, techniques, and preliminary compounds aimed at developing selective GDIs of $G\alpha$ subunits.

The TAT-GPR peptide and our small molecule GDIs were designed to stabilize the GDP bound conformation of $G\alpha_i$ by blocking nucleotide release and also interacting G-protein isoform specific residues around the nucleotide binding pocket. We determined that appending a TAT leader sequence was able to make the GPR peptide cell permeable without suppressing nucleotide exchange activity. Tagging with FGF was able to permeabilize the GPR peptide, but inhibited nucleotide exchange activity. We then determined that compared to PTX, TAT-GPR was very potent at preventing UK14304 stimulated decreases in cAMP.

Using computational docking we probed the $G\alpha_i$ -GDP binding using 280,000 diverse compounds. We also used $G\alpha_i$ -GTP and $G\alpha_q$ -GDP as computational counter-screens. From these simulations, we determined 210 potential $G\alpha_i$ -GDP interacting compounds. We desired to find compounds that stabilized the GDP-bound conformation of $G\alpha_i$ and therefore directly measured nucleotide exchange. The standard nucleotide exchange assay was modified by optimizing the emission bandpass to avoid fluorescence interference from test compounds. Fifteen compounds were tested in the first phase of validation and eight were active for $G\alpha_i$, although four were promiscuous and inhibited nucleotide exchange on $G\alpha_i$ and $G\alpha_q$. **0990CL**, **8770** and **2967** small molecular $G\alpha_i$ inhibitors showed varying degrees of efficacy to increase cAMP. From these data, a quinazoline scaffold emerged as tractable for $G\alpha_i$ inhibition. The structure-activity relationship (SAR) of the seven quinazoline derivatives indicated the critical need of both hydrophobic interactions provided by compound methyl groups and hydrogen bond donors provided by compound amine groups. We confirmed compound interaction by STD NMR. Data from the exchange assay, SAR,

NMR, and docking indicate that the 4'-Me moiety, and to a lesser degree, the 2'-Me moiety, from **0990CL** are involved in specific hydrophobic interactions. There are also significant compound interactions in the aromatic region and implicate the pyrimidine secondary amine participates in hydrogen bonding. The compound **0990CL** SAR did not indicate an obvious route to substantial improvement, and future characterization of hits such as **9595** and **9586** might provide better compounds in the long run. However, these compounds do demonstrate the feasibility of small molecule heterotrimeric Gα GDIs.

GDIs are well suited to pursue receptor pharmacology of Gα_i versus Gβγ signaling and their specific roles in cell signaling and disease.⁶⁹ A unique aspect of Gα–GDIs is their ability to selectively inhibit Gα with the potential to stimulate Gβγ signaling. This facet, along with Gβγ directed pharmacologic tools and PTX, will allow addressing the contribution of Gα, Gβγ, and Gαβγ signaling in living cells without alterations in protein stoichiometry. Several pharmacologic regulators of Gβγ have been developed including the SIGK peptide which dissociates the heterotrimer creating free Gα and Gβγ.^{70,71} The phosducin peptide inhibits Gβγ but allows Gα to signal.⁷² With these tools, pharmacologic regulation of both receptor-dependent and -independent signaling of individual heterotrimeric subunits can provide a unique window into this major signaling axis. Therapeutically, Gα subunit inhibition by GDI has the advantage of circumventing the need to directly address the upstream component of GPCR-related signaling in cases of mutations, polymorphisms, and expression-related defects often seen in disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AC	adenylyl cyclase
α_2AR	α_2 -adrenoceptor
cAMP	cyclic adenine monophosphate
Fsk	forskolin
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine diphosphate
GPCRs	G-protein coupled receptor
GPR	G-protein regulatory
GTP	guanine triphosphate
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
NMR	nuclear magnetic resonance
PTX	pertussis toxin
PLC	phospholipase-C
SAR	structure-activity relationship
STD	saturation transfer difference
Tc	Tanimoto coefficient

Figure 1.

The permeable GPR peptides and their effect of on GTP γ S binding to purified G α_i . (A) Amino acid sequences of GPR peptides. GPR motif consensus residues are colored in red and membrane permeable tags are colored in blue. (B and C) GTP γ 35S (500 nM) binding to purified G α_i 1 (100 nM) was measured in the absence and presence of peptides. Data are expressed as the percent of specific binding (~ 5 pmol) observed in the absence of peptide and represent the mean $\pm$ SD derived from two experiments performed in duplicate. The TAT-GPR data in B has been previously published.³⁸

Figure 2. Effect of $G\alpha_i$ inhibitors PTX and TAT-GPR on $\alpha_2AR/G\alpha_i$ mediated cAMP suppression. cAMP levels were measured in HEK293 GloSensor cells transfected with 0.2 $\mu\text{g/mL}$ α_2AR receptor. Cells were pre-treated prior to the experiment with 100 ng/mL PTX (24 h) or 0.1 μM and 10 μM TAT-GPR peptide (GPR, 20 min). Cells were subsequently treated with 250 nM Forskolin (Fsk) or Fsk and 10 μM of the α_2AR agonist UK14304. ** t Test between vehicle and TAT-GPR was significant with $p = 0.009$. Data was derived from triplicate determinates from three independent experiments.

Figure 3. Computational protein docking for the discovery of G-protein inhibitors. (A) Grid and placement spheres of $G\alpha_i$ used for site targeted docking simulations. (B) Ribbon diagram $G\alpha_i1-GDP-Mg^{2+}$. Ribbons are colored according to chain termini. (C) Top 210 compounds (from 280,000) rank ordered from ZINC neutral DOCK6 docking to $G\alpha_i1-GDP-Mg^{2+}$. (D) MOE based re-docking of top 210 compounds to $G\alpha_i1-GDP-Mg^{2+}$, $G\alpha_i1-GTP-Mg^{2+}$, and $G\alpha_q-GDP-Mg^{2+}$. Data shows scores for 30 poses per molecule.

Figure 4.

Primary validation of inhibitors by Gα_i versus Gα_q real-time fluorescence nucleotide exchange assay. (A and B) For each experiment 600 nM Gα_i or Gα_q was preincubated with vehicle (grey and red) or 300 μM (green), 100 μM (purple), or 30 μM (olive) test compound **0990CL** for 30 min prior to the addition of BODIPY FL GTPγS, and nucleotide exchange was monitored via fluorescence for 60 min. Background signals were determined using BODIPY FL GTPγS alone and is represented in black. Unlabeled GTPγS introduced to control reactions after 20 min (red) resulted in a decrease in fluorescence through time and demonstrates the nucleotide exchange viability of G-protein. (C and D) Endpoint results (60 min) of the kinetic assays depicted in A and B for Gα_i and Gα_q for compounds **4630**, **8005**, and **0990CL** on Gα_i and Gα_q. Data in C–F was derived from triplicate determinations from three independent experiments.

Figure 5. Saturation transfer difference (STD) NMR of Gα_i1 and **0990CL**. (A) Chemical graph depicting inhibitor compound **0990CL**, proton numbering scheme for STD NMR and T₁-relaxation times (italic). (B) Enlargement of the aliphatic region of the reference and STD NMR spectrum of **0990CL** with Gα_i. Increases in STD signals are observed with saturation times (τ_{sat}) of 1 s (blue) and 3 s (red) and **0990CL** proton assignments are indicated. (C) Tautomer Gα_i inhibitor **0990** docked with Gα_i GDP. T₁-compensated STD effects observed using τ_{sat} = 3 s were normalized to H3' which was set to 100%. (D) Enlargement of the aromatic region of the reference and STD NMR spectrum of **0990CL** with Gα_i. Increases in STD signals are observed with saturation times (τ_{sat}) of 1 s (blue) and 3 s (red) and **0990CL** and H8(GDP)-proton assignments are indicated.

Figure 6.

Effect of small molecule $G\alpha_i$ inhibitors on $\alpha_2AR/G\alpha_i$ mediated cAMP suppression. cAMP levels were measured in HEK293 GloSensor cell line was transfected with 0.2 $\mu g/mL$ α_2AR receptor. Cells were pre-treated prior to the experiment with 100 ng/mL PTX (24 h), 0.1, and 1 μM **0990CL** (20 min) or 0.1, 1, and 10 μM **8770**, **2967**, and **2355** (20 min). Cells were subsequently treated with 250 nM Fsk or Fsk and 10 μM of the α_2AR agonist UK14304. Data was derived from triplicate determinates from three independent experiments.

Table 1
Primary screening for GDIs through chemical diversity sampling

CAS #	ID		% Inhibition	
			Gα _i	Gα _q
864389-67-3	4630		38	<10
907989-49-5	9827		35	<10
511514-03-7	0990		25	<10
849019-55-2	1217		30	<10
301353-19-5	7136		13	<10
749837-97-6	7507		<10	<10
1070463-59-0	8005		<10	27

CAS #	ID		% Inhibition	
			Gα _i	Gα _q
284029-69-2	1254		<10	–
1020944-29-9	1174		<10	–
1184184-00-6	2967		34	50
1244948-00-2	6715		38	35
312609-02-2	1026		18	40
111015-95-3	8770		<10	32
712346-46-8	4799		<10	25