Functional characterization of Gαi/s proteins and modulation of their activity by linear and cyclic peptides

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Thessaloniki, Griechenland

Bonn, August 2023

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 15.01.2024
Erscheinungsjahr: 2024

‘‘The eye cannot see what the mind does not know.’’

~ modified from *Henri Bergson* ~

Abstract

Heterotrimeric Gαβγ (guanine nucleotide-binding) proteins serve as molecular switches to activate ("switch on") or inactivate ("switch off") diverse intracellular signaling cascades in response to stimulated G protein-coupled receptors (GPCRs). Modulation of these G proteins can be impelled by developing chemical tools, such as bioactive peptides. Such compounds are able to interact with distinct binding sites in the protein and inhibit protein-protein interactions. This dissertation reviews and outlines two main objectives, i) the functional characterization and activation mechanism of the Gαi1, Gαs(long), and Gαs(short) protein subunits, and ii) the development of linear and macrocyclic compounds with biological significance for the Gαi/s protein modulation. Basic computational analyses including molecular docking and dynamics simulations assisted the underlying studies.

The first aim of the present thesis is to comprehend the molecular basis for the activation of the Gαi/s protein subunits in the context of guanine nucleotide binding. The acquisition of the functional active protein substantially contributes to Gαi/s protein characterization and modulation. The determination of the active fraction of the Gαi1 and Gαs proteins and the elucidation of the biochemical differences of the Gαs(long) and (short) isoforms, which was paused for decades, pave the way for the future analysis of other Gα protein subunits. Both novel and already known non-radioactive fluorescently labeled guanine nucleotide analogs are utilized for the functional analysis and characterization of the enzymatic activity employing fluorescence, fluorescence anisotropy and GTPase-based assays.

This leads to the second main focus of this work, i.e., the development of potent and specific linear and macrocyclic peptide modulators targeting either the Gαi and/or Gαs protein subunit. Peptides derived from a one-bead-one compound (OBOC) combinatorial peptide library, such as the lead compound GPM-1 (and derivatives) exhibited a GEM (guanine exchange modulator)-like activity for the Gαi/s·GDP, i.e., the inactive protein state. A series of bicyclic compounds from a one-bead-two compound (OBTC) peptide library reveal GPM-3 as the first peptide acting as a class- and state-specific GAP (GTPase-activating protein)-like modulator of Gαi1·GMPPNP, and GPM-2 as a potential GEF (guanine nucleotide exchange factor) modulator of Gαi1·GDP.

Thus, these objectives and the results thereof are extensively described in the following chapters (the respective manuscripts are enclosed in the appendix) and could act as the stimulus for the biochemical investigation of other classes of Gα proteins either with nucleotide analogs and/or with bioactive peptides, assessing the potential druggability of G proteins in general, as well as of Gαi and Gαs in particular.

Zusammenfassung

Heterotrimere Gαβγ-Proteine (Guanin-Nukleotid-bindende Proteine) dienen als molekulare Schalter zur Aktivierung ("Einschalten") oder Inaktivierung ("Ausschalten") verschiedener intrazellulärer Signalkaskaden als Reaktion auf stimulierte G-Protein-gekoppelte Rezeptoren (GPCRs). Die Modulation dieser G-Proteine kann durch die Entwicklung chemischer Werkzeuge wie bioaktiver Peptide vorangetrieben werden. Diese Verbindungen sind in der Lage, mit bestimmten Bindungsstellen im Protein zu interagieren und Protein-Protein-Wechselwirkungen zu hemmen. In dieser Dissertation werden zwei Hauptziele untersucht und dargestellt: i) die funktionelle Charakterisierung und der Aktivierungsmechanismus der Gαi1-, Gαs(lang)- und Gαs(kurz)-Proteinuntereinheiten und ii) die Entwicklung von linearen und makrozyklischen Verbindungen mit biologischer Bedeutung für die Modulation von Gαi/s-Proteinen. Die zugrundeliegenden Studien wurden durch grundlegende rechnerische Analysen einschließlich molekularem Docking und Dynamiksimulationen unterstützt.

Das erste Ziel der vorliegenden Arbeit ist es, die molekulare Grundlage für die Aktivierung der Gαi/s-Proteinuntereinheiten im Zusammenhang mit der Guaninnukleotidbindung zu verstehen. Die Erfassung des funktionellen aktiven Proteins trägt wesentlich zur Charakterisierung und Modulation des Gαi/s-Proteins bei. Die Bestimmung des aktiven Anteils der Gαi1- und Gαs-Proteine und die Aufklärung der biochemischen Unterschiede der Gαs(lang)- und (kurz)-Isoformen, die jahrzehntelang pausiert wurde, ebnen den Weg für die zukünftige Analyse anderer Gα-Protein-Untereinheiten. Sowohl neuartige als auch bereits bekannte nicht-radioaktive fluoreszenzmarkierte Guaninnukleotid-Analoga werden für die funktionelle Analyse und Charakterisierung der enzymatischen Aktivität unter Verwendung von Fluoreszenz, Fluoreszenzanisotropie und GTPase-basierten Assays verwendet.

Dies führt zum zweiten Schwerpunkt dieser Arbeit, d. h. der Entwicklung wirksamer und spezifischer linearer und makrozyklischer Peptidmodulatoren, die entweder auf die Gαi- und/oder Gαs-Proteinuntereinheit abzielen. Peptide, die aus einer kombinatorischen One-Bead-One-Compound (OBOC)-Peptidbibliothek stammen, wie die Leitverbindung GPM-1 (und Derivate), zeigten eine GEM (Guanin-Austausch-Modulator)-ähnliche Aktivität für Gαi/s·GDP, d. h. den inaktiven Proteinzustand. Eine Reihe bizyklischer Verbindungen aus einer OBTC-Peptidbibliothek (One-Bead-Two-Compound) zeigt GPM-3 als erstes Peptid, das als klassen- und zustandsspezifischer GAP (GTPase-aktivierendes Protein) -ähnlicher Modulator von Gαi1·GMPPNP wirkt, und GPM-2 als potenzieller GEF (Guaninnukleotid-Austauschfaktor) -Modulator von Gαi1·GDP.

Diese Ziele und ihre Ergebnisse werden in den folgenden Kapiteln ausführlich beschrieben (die entsprechenden Manuskripte befinden sich im Anhang) und könnten als Anregung für die biochemische Untersuchung anderer Klassen von Gα-Proteinen entweder mit Nukleotidanaloga und/oder mit bioaktiven Peptiden dienen, um die potenzielle Medikamentenverfügbarkeit von G-Proteinen im Allgemeinen sowie von Gαi und Gαs im Besonderen zu bewerten.

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1. Introduction and thesis outline

The heterotrimeric guanine nucleotide binding proteins, referred to as G proteins (Gαβγ), are vital key players in the intracellular signal transduction[1]. The discovery of the G proteins and their implication to cellular signaling pathways was acknowledged with a Nobel Prize in Physiology or Medicine in 1994 to Alfred Gilman[2] and Martin Rodbell[3]. Their physiological function is highly associated with the targeted cell or tissue type. Membrane-bound G proteins relay information from an extracellularly stimulated (hormones, neurotransmitters etc.) G protein-coupled receptor (GPCR). The relevance of GPCRs in medicinal chemistry has been long recognized, since approximately 30 – 40% of the FDA-approved drugs address these receptors[4–6]. Mutations or malfunctions of several GPCRs were reported to lead to severe diseases, such as asthma and cancer[7,8]. Moreover, upon GPCR stimulation, G proteins are activated leading to heterotrimer dissociation to the Gα monomer and the Gβγ dimer. Each moiety can subsequently induce downstream signaling cascades via interaction either with effectors or accessory proteins. Despite the limiting localization of G proteins (deficient accessible sites within the cell surface), dysregulation of their activation was tightly correlated with various pathophysiological conditions (cancer, heart failure, inflammatory diseases etc.[6,8]). Recently, several approaches were conducted in order to assess the druggability of G proteins (especially of Gα subunits) in a GPCR-independent manner by developing pharmacological tools[9–12].

The prerequisite of the common G protein activation is the initiation of the so-called GTPase-cycle, in which the guanosine diphosphate (GDP)-bound Gα protein is exchanged to the guanosine triphosphate (GTP) analog. The GDP dissociation and GTP binding is the rate limiting step of the above cycle and determines the G protein fate[13]. To date, primarily radioactive and relatively indirect assays were applied to analyze these phenomena lacking though the accurate Gα protein activity determination[14–17]. Therefore, the extensive investigation of the Gα-protein's ability to bind GTP is crucial. Following the non-canonical Gα protein modulation pattern and considering that G proteins can transmit cellular responses through protein-protein interactions (PPIs), peptides have been broadly utilized as chemical probes to modulate the G protein-mediated signal transduction[9, 11,18–20]. Chemical modifications, such as (macro)cyclization, amino acid derivatization etc. have been shown to pharmacologically improve the cell permeability and subsequently function of the ligands[18,19,21–24]. The present thesis provides an efficient strategy to quantitatively determine the activity of Gαi/s proteins and their subsequent modulation with linear and (macro)cyclic peptidyl modulators.

1.1 Structure and function of heterotrimeric G proteins

A cell is capable of receiving plentiful extracellular signals and induces a specific response to these stimuli via different receptor families and their respective signaling pathways. One of the largest families of membrane proteins are the GPCRs, including more than 800 different proteins[6–8]. Upon interaction of these seven transmembrane proteins with an exterior ligand (hormones, neurotransmitters etc.), the GPCRs are activated. In all eukaryotic organisms, heterotrimeric Gαβγ proteins in response to an activated GPCR could act as molecular binary switches for subsequent intracellular signaling cascades,

such as cell growth, motility, and survival[25]. In response to GPCRs, G proteins undergo some conformational changes that allow the induction of distinct signal transduction mechanisms targeting several tissues.

At the native heterotrimer structure, G proteins consist of the Gα (39 – 52 kDa), the Gβ (35 – 36 kDa) and Gγ (8 kDa) subunits[26]. The Gβ and Gγ polypeptides are tightly associated with each other and function usually as one unit or commonly known as a Gβγ dimer[27]. Interestingly, the variable homology of heterotrimeric G proteins is supported by 35 different genes, among them 16 Gα- (Figure 1), 5 Gβ- and 14 Gγ- encoding genes[26–30]. The focus of this dissertation is on the Gα subunit, the largest subunit of the heterotrimeric G proteins, which consists of the Gαs/olf (Gαs(long)/(short), Gαolf), Gαi/o (Gαi1-3, Gαt1-3, Gαo1-2, Gαz), Gαq/11 (Gαq, Gα11, Gα14, Gα15) and Gα12/13 (Gα12, Gα13) protein subfamilies. The Gα subunit is a vital player and determinant of the G protein signaling, because of its

Sequence similarity of mammalian Gα protein subunits

Subunit		Gαolf	Gαi1	Gαi2	Gαi3	Gαo	Gαt1	Gαt2	Gαgust	Gαz	Gαq	Gα11	Gα14	Gα15	Gα12	Gα13
Gαs/olf	Gαs	77	40	40	40	42	39	40	41	37	39	40	39	35	36	36
	Gαolf		41	41	40	42	42	42	42	38	39	39	38	35	36	37
Gαi/o	Gαi1			87	93	72	67	69	67	67	50	51	51	43	42	39
	Gαi2				85	68	65	69	66	66	50	50	50	43	41	40
	Gαi3					66	61	65	62	65	50	48	48	41	39	36
	Gαo						61	61	61	59	50	49	50	41	43	42
	Gαt1							78	75	53	52	51	48	44	41	38
	Gαt2								79	57	51	50	49	43	41	40
	Gαgust									57	51	50	50	41	41	40
	Gαz										49	48	48	41	41	39
Gαq/11	Gαq											84	76	54	41	43
	Gα11												82	56	43	44
	Gα14													54	42	44
	Gα15														40	38
Gα12/13	Gα12															67

Figure 1: Sequence similarity between the 16 genes identified for the different mammalian Gα protein subfamilies. The numbers indicate the percentage (%) of amino acid identity from BLAST (NCBI). The figure was modified from Downes *et al*.[29,31] (red: sequence similarity < 50%, green: sequence similarity > 60%, black frames highlight the comparison between Gαs and Gαi subfamilies).

ability to bind and hydrolyze guanine nucleotides. At the native and inactive state, the Gα shows high binding affinity to GDP and keeps the heterotrimer structure intact (Figure 2). After G protein activation, GDP is exchanged to GTP, which subsequently leads to heterotrimer dissociation into the active Gα·GTP monomer and the Gβγ dissociated moiety[32]. Both subunits can individually target downstream effectors and stimulate distinct signaling pathways in an independent, synergistic, or antagonistic manner[33]. However, the Gα protein subunit attains an intrinsic GTPase activity promoting the deactivation and eventual termination of the described above, GTPase cycle. Once the GTP is hydrolyzed to GDP, the heterotrimeric Gαβγ is reassociated (Figure 2) and the signal transduction is terminated[1, 13,25].

In order to identify the source of this vital ability of the G protein to induce cellular signaling, the scientific community resolved significant structural properties of the protein. The Gα subunit consists of an α-helical domain (AHD) and a GTPase (Ras-like) domain. In between, there is a cleft that exhibits high binding affinity for the respective guanine nucleotide. The nucleotide binding to this pocket is stabilized by various loops, linkers, and switch (SW) regions as will be further described below (chapter 1.1.2). These regions, and especially the SW(I-III) regions[34–36], are involved in the G protein activation due to significant conformational changes enabling the heterotrimer dissociation.

The fact that more than 30% of the FDA-approved drugs address GPCRs[4] in combination with the close relation between GPCRs and G proteins, with the latter one being involved in cell viability, raised the interest in investigating in-depth the G protein signal transduction. Comprehending the function and structure of the Gα protein subunits is the stepping-stone for targeting these proteins as pharmacological tools.

1.1.1 Signal transduction pathways of G proteins

The fundamental principle of the signaling mechanism is the transmission of an extracellular message to the intracellular machinery. The G protein-mediated signaling mechanism takes place in all eukaryotes. At the canonical G protein activation[37–39], a GPCR-agonist binds to the receptor and forms a ternary complex with the heterotrimeric Gαβγ protein, where, at the basal ("off") state, a GDP nucleotide is bound to the Gα subunit (Figures 2, 3). Conformational changes taken place within the Gα subunit enable the exchange of GDP into GTP, which is intracellularly more abundant compared to its GDP counterpart[40]. The GPCR-G protein interface is approx. 30 Å, thus canonical G protein activation via GPCRs leads to structural changes in the protein and subsequent GDP release[5]. The empty-pocket (nucleotide absence) state is highly dynamic indicating the rapid nucleotide exchange and initiation of the G protein cycle[41]. The release and subsequent binding of GTP to the Gα subunit results in heterotrimer dissociation and G protein activation. Thereby, the heterotrimeric G protein is detached from the membrane bound GPCR and then internally dissociated into two functional units, the Gα subunit and the Gβγ dimer.

The question on how the signal or information is transmitted intracellularly to different tissues is resolved through effector molecules, such as enzymes, proteins or even ion channels[42–44]. The C-terminus of the protein interacts with the receptor, whereas the N-terminus exhibits affinity to effectors, functioning as a communication-bridge between the membrane and the cell interior[45,46]. Each G protein subclass interacts with distinct downstream effectors emphasizing the heterogeneity of the G proteins. Figure 2 summarizes important effector molecules involved in G protein signaling. For instance, the members of Gαs/olf subfamily (stimulatory G proteins) showed significant interactions with all adenylyl cyclase (AC) isoforms AC(I-IX)[27,47] upon activation of the β_2-adrenergic (β_2-AR) receptor[48–50]. Thus, this interaction facilitates the subsequent stimulation of second messengers, such as cyclic adenosine monophosphate (cAMP) via adenosine triphosphate (ATP) and protein kinase A (PKA)[27,51–56]. In contrast, members of the Gαi/o family evoke the inhibition of the AC and subsequently of cAMP[57–59]. Therefore, the activation

of e.g., the D2L dopamine (Gαo[59]), μ-, δ-, or κ-opioid (Gαi/o[60,61]) receptor induces the Gαi/o-protein (inhibitory G proteins)-mediated signaling. The Gαt transducin has been found to interact with rhodopsin and subsequently stimulates the retinal phosphodiesterase (PDE)[27]. Moving on to a different signaling pathway, two members of the Gαq/11 (Gαq and Gα11) subfamily can interact with the activated α_1-AR or angiotensin (AT1) receptors and activate the β or γ isoform of phospholipase C (PLC)[62,63]. Subsequently, DAG (diacylglycerol) and IP_3 (inositol 1,4,5-trisphosphate) are secreted in order to interact with protein kinase C (PKC) or with Ca^{2+} channels, respectively[26,27,64,65]. The intracellular function of the Gα14 and Gα15 subunits is still unknown[63]. The fourth and final Gα protein subclass, the ubiquitously expressed Gα12/13 subfamily[28], is tightly associated with small GTPases[25, 62,66], such as

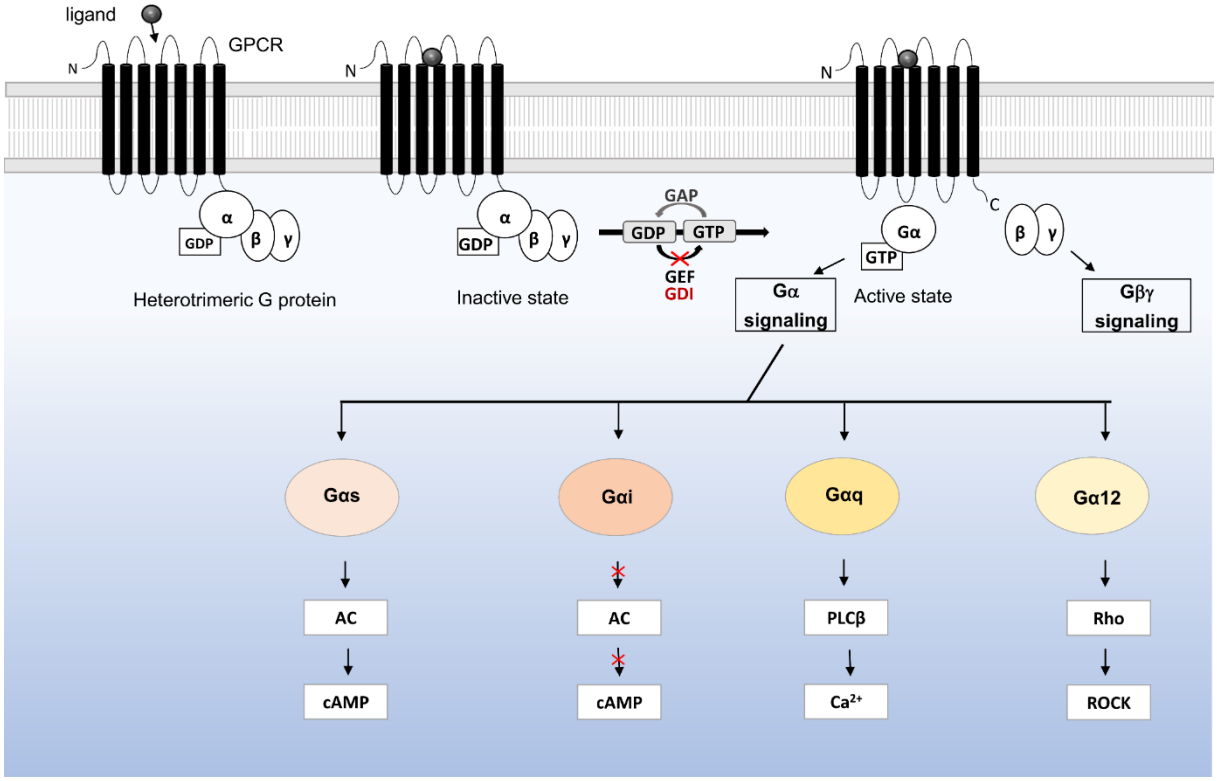

Figure 2: Signal transduction mechanism of heterotrimeric G proteins. Upon ligand binding to G protein-coupled receptors (GPCRs), heterotrimeric G proteins are activated by exchanging the GDP nucleotide for the triphosphate analog GTP. This leads to dissociation into the monomeric GTP-bound Gα subunit and the Gβγ dimer. Each individual moiety is responsible for the downstream activation of intracellular effector molecules. The Gαs subfamily stimulates the activation of adenylyl cyclase (AC) and subsequently the formation of cAMP, whereas Gαi acts in the opposite manner. The Gαq subunit activates the phospholipase Cβ (PLCβ) involved in the activation of calcium channels. Finally, the Gα12 subunit is closely related to the Rho-mediated signaling. Accessory proteins can enhance (GEF: guanine nucleotide exchange factor) or prevent (GDI: guanine dissociation inhibitor) the G protein activation. Signal termination is facilitated via GAPs (GTPase-activating proteins). This figure was inspired and modified from reference[5].

the Rho- or Rac-dependent signaling mechanisms via interaction with the p115-RhoGEF effector[62,67]. Activation of Rho leads to stimulation of the Rho-associated protein kinase (ROCK) pathway. The actin filament and cytoskeleton rearrangements are some of the key roles of these proteins[63,67–72]. With respect to the Gβγ dimer, important interaction partners are the acetylcholine/GIRK (G protein-regulated

inwardly-rectifying potassium) channel, some AC isoforms and indirectly, the MAP kinase[57,58,73–79]. Aside from the canonical G protein activation mechanism, a non-canonical mode can also be initiated (Figure 3)[37,38,80,81]. In this non-classical process, three different categories of molecules are primarily involved: the GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), and GDIs (GDP dissociation inhibitors)[32,82]. Moreover, G proteins can directly interact with adhesion proteins or microtubules intracellularly[38], or assisted by accessory regulatory proteins, such as members of the receptor-independent AGS (activators of G protein signaling[83–85]) and RGS (regulator of G protein signaling[86–89]) family or chaperone proteins, such as Ric-8A/B[90–96]. Since all the aforementioned proteins modulate the G proteins individually, their functions will be described in the following chapter (1.3). In conjunction with the above points, all Gα proteins were reported to have a medicinal relevance for various diseases. For instance, Gαs has been involved in breast[97,98] and pancreatic cancer[56,99], and

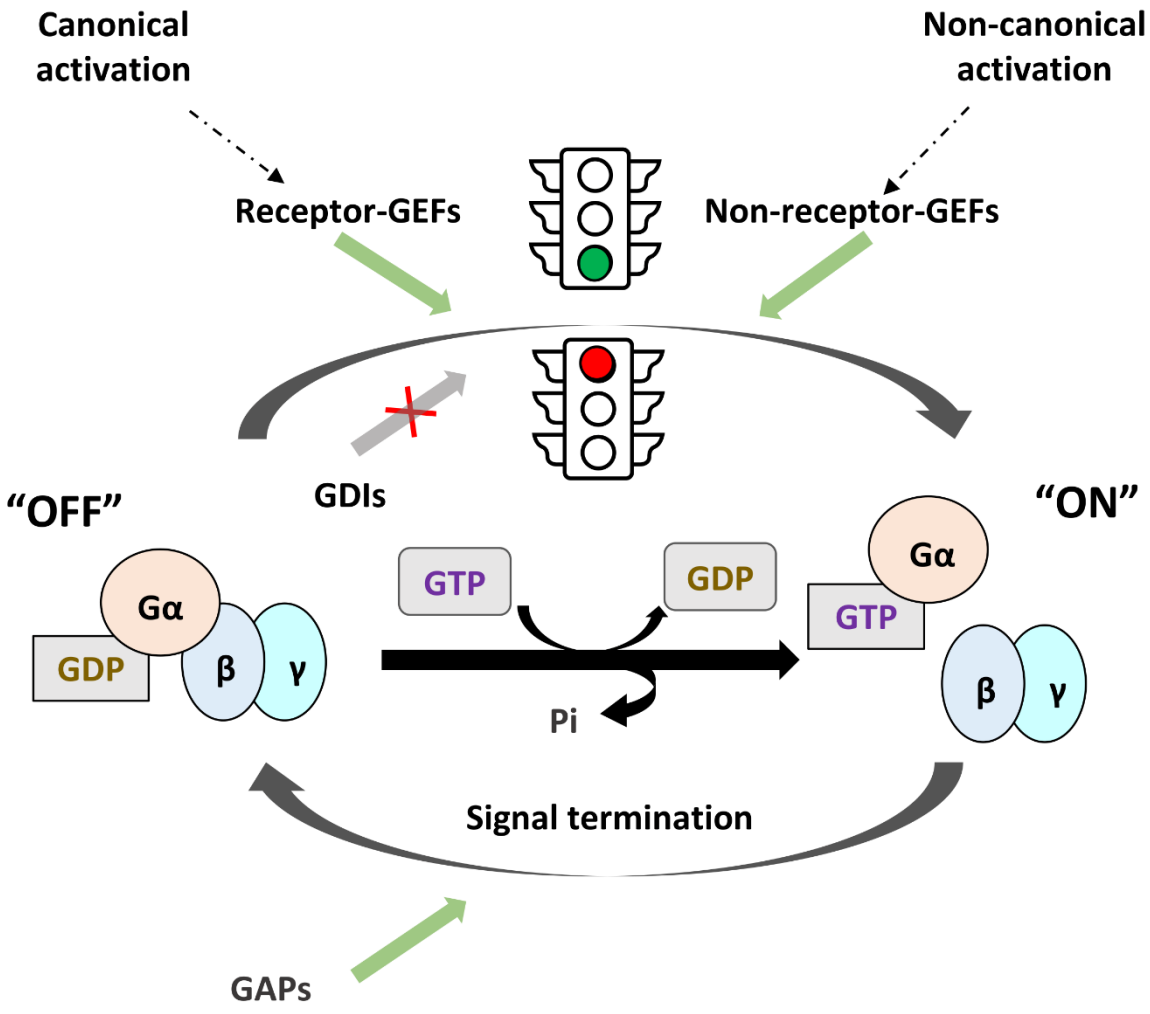

Figure 3: The GTPase cycle with canonical and non-canonical G protein activation signaling with accessory protein molecules. GEFs enhance the GDP dissociation and GTP binding, switching on the GTPase cycle. GEFs associated with GPCRs represent the canonical activation pathway, whereas GEFs directly targeting the G proteins, initiate the non-canonical signaling. GDIs hinder the nucleotide exchange keeping the GDP nucleotide intact on the Gα subunit (inactive state). The GTPase cycle can be terminated with GAPs promoting the GTPase hydrolysis. The figure was inspired from earlier publications[80,100,101].

endocrine disorders[102,103], Gαi in heart failure[104,105], thrombosis[106–108] as well as breast cancer[97,109], Gαq/11 is tightly involved in uveal melanoma,[110–112] and Gα12/13 was found to promote a plethora of oncogenic dysregulations[62, 68,71] (e.g., lymph nodes[69], prostate[70,113], kidney,[114] and liver[115]). Remarkably, out of around 130 approved GPCRs-targeting drugs, the majority is addressing Gαi/oPCRs (45%) and GαsPCRs (30%)[4], whereas no G protein drug has been tested or approved so far[8,116,117].

1.1.2 Structural analysis of G proteins

Since the beginning of 1990[34,118], essential structural studies were performed in order to investigate in detail the function and molecular mechanisms of the heterotrimeric G proteins. As stated above, all Gα subunits consist of two domains, the AHD and the GTPase domain and in between these regions there is a conserved cleft[119] exhibiting high binding affinity for guanine nucleotides, GDP at the inactive and GTP for the active protein state[31]. Therefore, the core moiety determining the G protein signaling is the guanine nucleotide. Since G proteins are also characterized as molecular switches, the source of this mechanism was examined. The rapid intrinsic GTPase activity of the G proteins restricts the stability of a transition or empty-pocket state upon nucleotide exchange[86,119,120]. The first crystallographic study uncovered the atomic interactions of the Gαt protein with the GTP-analog, GTPγS (PDB ID: 1TND[35]), followed by the structure of the respective inactive state, where GDP was bound to the protein (PDB ID: 1TAG[34] and 1GOT[121], Figure 4a). X-ray crystallography, Cryo-EM (cryogenic electron microscopy) and NMR (nuclear magnetic resonance) experiments have been efficiently conducted resulting in structural resolution of Gα-guanine nucleotide[34,35, 44,122–125] and GPCR-G protein complexes[48,126–130]. Based on these models, conformational changes occur on three switch regions (SWI-III, Figure 4b), upon activation of heterotrimeric G proteins, which are responsible for switching “on” and “off” the G protein-

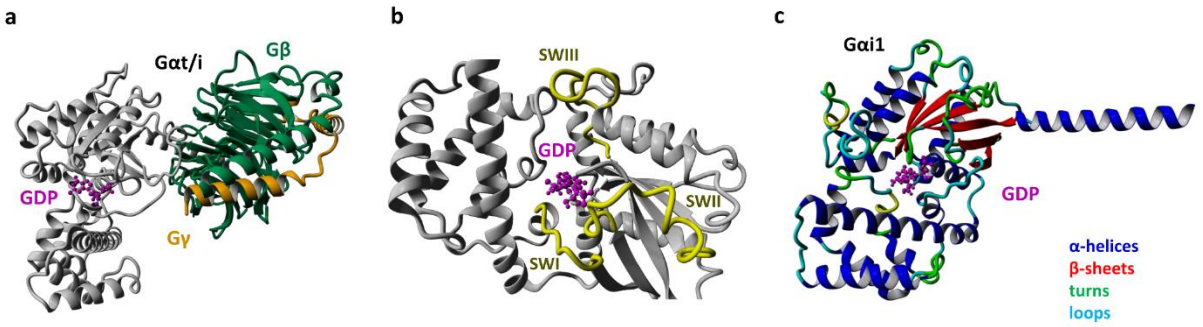

Figure 4: Structures of G proteins. (a) Crystal structure of the heterotrimeric Gαt/iβγ protein (PDB ID: 1GOT[121]) in the inactive state (GDP nucleotide in magenta). The Gβ and Gγ domains are presented in green and orange colors, respectively. (b) The Switch regions (SWI-III) are highlighted in yellow in the homology model (HM) of Gαi1·GDP, which was described in reference[21] (PDB IDs: 3UMS[131], 5JS8[125], and 1Y3A[12]). (c) Overview of α-helices (in blue), β-sheets (in red), turns (green), and loops (cyan) of the Gαi1·GDP HM.

-mediated signaling[118,119,132]. Also, the GTPase domain contains six stranded β-sheets (β1-β6) and five α-helices (α1-α5), the phosphate binding loop (P-loop) and the binding sites for Mg^{2+} ions (Figure 4c). The two main domains of the Gα subunit are connected with each other with two linkers (linker I and linker II). The AHD has a prevailing α-helical character regarding the secondary structure of the

protein[125,133,134], with a central and long αA-helix and five further α-helices (αB-αF)[34,35]. The high flexibility and dynamic behavior of the AHD has been long known[126,135].The residues 65-150 of AHD are conserved in all G protein subunits except for the Gαs(short), and α3-β5 and α4-β6 loops (Figure 5a,b) are distinguishably different between Gαs(short) and Gαi subunits implying their significance concerning the subsequent downstream effector binding[44].

A deeper look into the nucleotide binding pocket demonstrates the prerequisite of the Mg^{2+} ion as a stabilizer of the nucleotide and the function of five structural motifs in close proximity to the nucleotide binding cavity. The TCAT and N/TKXD motifs facilitate the binding of the guanine base and the GDP stabilization[5]. The phosphate binding in the P-loop is mediated by the GXGESGKST sequence. Lastly, the RXXTXGI and DXXG motifs provide residues for Mg^{2+} binding to the SWII region[5, 34,35, 132,136,137]. During the canonical G protein activation mechanism, conformational rearrangements of TCAT affect the β6-α5[138] and β1-α1 loop[139], reduce hydrophobic interactions of α5 and thus disrupt the function of the P-loop. This causes a very dynamic change of the nucleotide pocket where GDP reduced its associ-

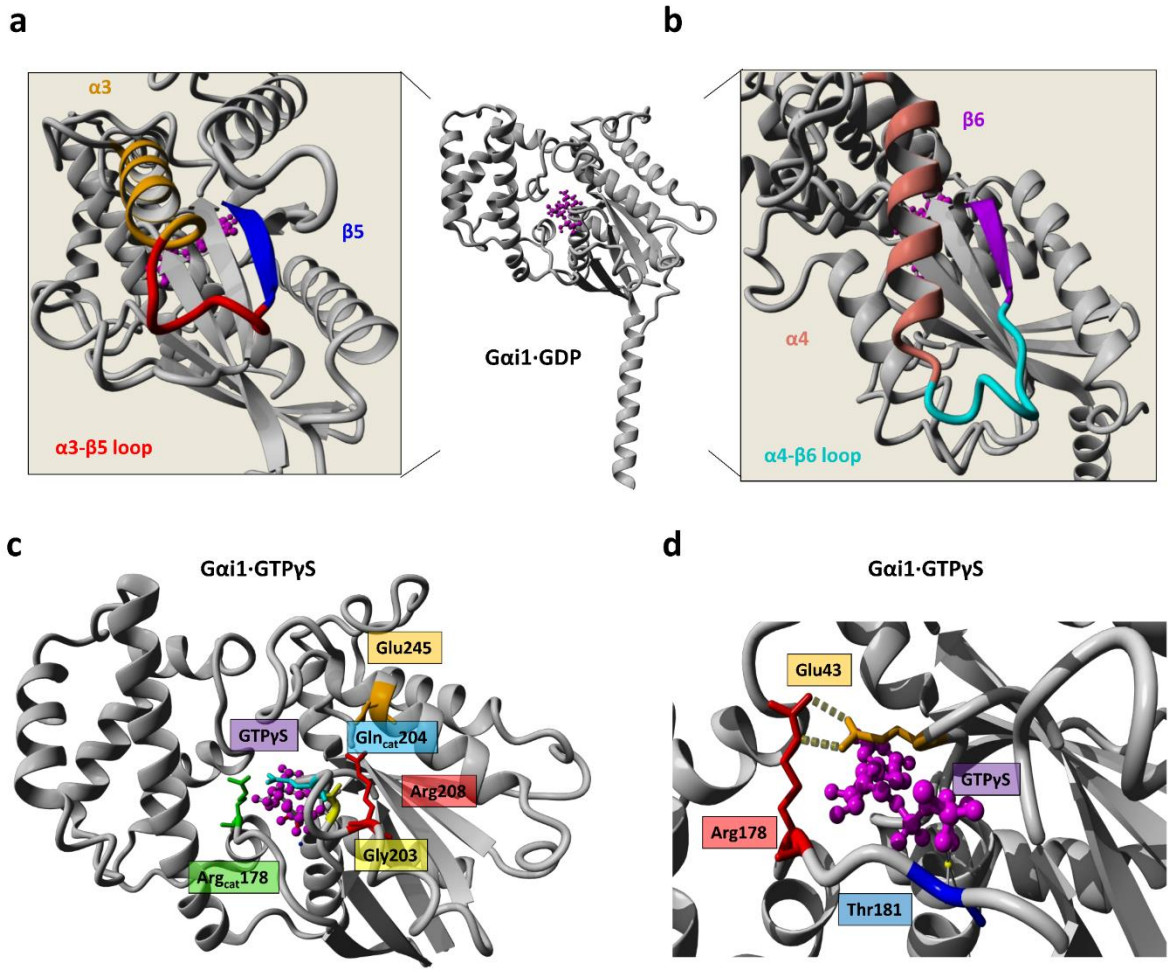

Figure 5: Catalytical regions and residues for Gα protein function. (a, b) Depiction of α3-β5 and α4-β6 loops of HM[21] Gαi1·GDP protein subunit. (c) Crystal structure of Gαi1·GTPγS (PDB ID: 1GIA[122]) showing the G-R-E triad (Gly203, Arg208, and Glu245). (c,d) Two catalytic for GTP hydrolysis residues (Arg178 and Gln204) and the salt bridge between Arg178 and Glu43 in the P-loop are depicted. Panels (c, d) are modified from Pepanian *et al*.[140].

-ation with the two domains facilitating the final GDP release[119,132,141]. Upon binding of the highly concentrated cytosolic GTP[122], SWI and SWII regions are primarily involved with the two residues Thr35 (SWI) and Gly60 (SWII) within the DXXG motif (both identified for the Ras protein) allowing the binding of the γ-phosphate and the Mg^{2+} ions via the side chain of Thr35, stabilizing the GTP binding in the cavity[132]. Moreover, alterations in the SWI region (Ser173–Thr183) contribute to the heterotrimer dissociation (interaction with the Gβγ unit) via hydrogen bonding formation of Arg174 and Thr177 with the γ-phosphate of the GTP molecule[133]. This is known as the "push and pull" effect, where Mg^{2+} is pushed from its normal place along with SWI, whereas SWII seems to be pulled closer to the guanine nucleotide[132]. In parallel, Gly199, which is residing in SWII (Phe195–Thr215), gets in contact with the γ-phosphate of GTP. Then, the hydrogen bond between the β3-β1 is disturbed and new contacts between α2 (Arg201, Arg204, and Trp207), and α3 (Glu241, Leu245, Ile249, and Phe255) are formed[35,142]. Additionally, a new polar network between SWII and SWIII (Asp227–Arg238) through interactions of the aforementioned Gly199, Arg201, and Arg204 with Glu232 is established[35,143]. Recently, a new unit, called G-R-E triad, consisting of Gly203, Arg208, Glu245 (Figure 5c) stabilizes the tight interaction between SWII and SWIII, keeps GTP binding stable and thus promotes the Gβγ dimer release[144]. Additional interactions with molecules, such as GEFs[136,145,146] or other effectors[51,147,148] have also been resolved, and revealed that interaction of the Gαs(short) with its effector AC was facilitated through the α3-β5 and α4-ββ6 loops[44,149]. In spite of the activation of the G proteins with the heterotrimer dissociation, an important aspect to examine is also the termination of the signal or in other words, the GTPase hydrolysis mechanism and subsequent re-association of the Gαβγ complex. Three main residues, Arg178 (arginine finger or catalytical arginine, residue numbering according to Gαi1 protein subunit[137]), Gln204 (catalytical glutamine) and Thr181, are involved (Figure 5d, 6). The catalytical Arg178 can also establish a salt bridge with the P-loop (Glu43) directing the gradual GDP binding (Figure

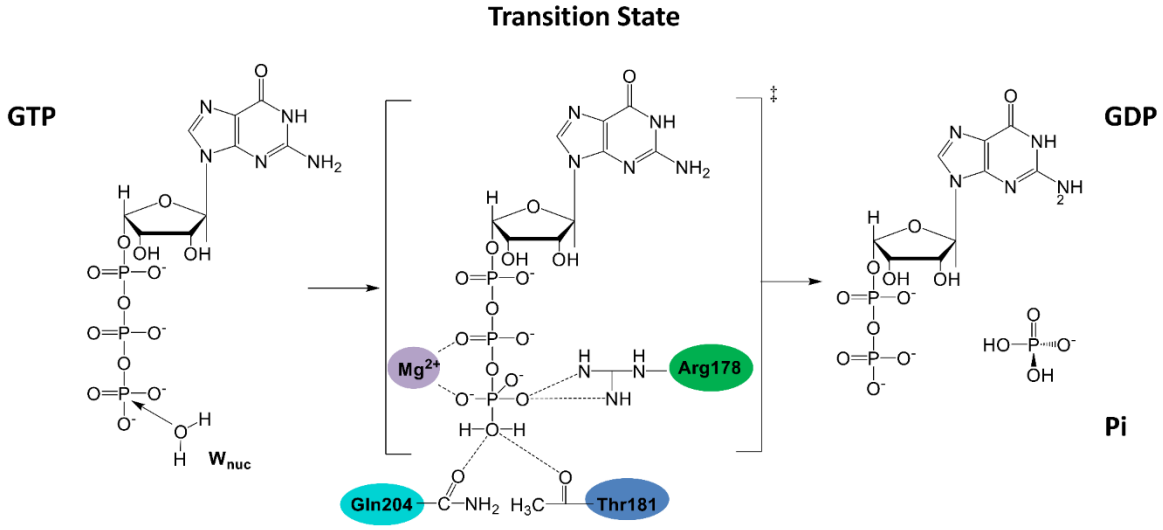

Figure 6: Proposed mechanism of GTP hydrolysis. Nucleophilic attack of the water molecule (W_{nuc}) facilitated by Gln204 (in cyan) and Thr181(in blue) to cleave the bond between the γ– and β–phosphate[122]. Bond disruption between the catalytical Arg178 (in green) and the γ–phosphate promotes the GTP hydrolysis[36,144]. Illustration of the mechanism modified from Coleman *et al*.[122].

5d)[12,121,150]. Thr181 and Gln204 facilitate the nucleophilic attack of the water molecule (W_{nuc}) for the hydrolysis of GTP into GDP with the bond cleavage between the γ– and β–phosphate (Figure 6)[36,131]. Once the GTP is intrinsically, or assisted by GAPs, hydrolyzed, the SWI is slowly moving away from the nucleotide binding pocket with diminished Mg^{2+} ion interactions. Different RGS accessory proteins, such as RGS4 have been identified to promote GTP hydrolysis and provided structural information to comprehend this mechanism[36,89,120,151,152]. The network between SWII-SWIII (G-R-E triad) is disturbed due to conformational rearrangements of SWII removing Gln204 from the catalytic site that in turn favors the heterotrimer re-association and Pi (inorganic phosphate) release[143,153].

A further interesting structural determinant of the heterotrimeric G protein activation is the interface between the Gα subunit with the Gβγ dimer. Lipid modifications (myristoylation or palmitoylation) of Gα and prenylation of Gγ subunits stabilize them on the inner site of the plasma membrane and enhance the interaction between the two units[154]. The Gβ subunit attains a sequential motif of the WD proteins (WD40 motif) and belongs to the β-propeller protein containing 7 blades and 4 antiparallel β-sheets (Figure 7a)[117,154–156]. The contact between the Gα subunit and the Gβγ dimer is mediated by bivalent interactions between Gα and Gβ (αN-helix and β1-sheet, Figure 7b), without direct contact between Gα and Gγ[157]. This structure resembles a propeller-like shape and explains the origin of the name. Crystallographic structures of the Gβγ dimer of transducin (PDB ID: 1TBG)[156], Gαt/iβ1γ1 (PDB ID: 1GOT)[121], and Gαiβ1γ2 (PBD ID: 1GP2)[155] revealed that the N-terminal helix of Gβ interacts with the N-terminal helix of the Gγ subunit establishing a very stable complex, while the WD40 repeat is associated with the SWII region of the Gα (as a heterotrimer) or with different effector molecules upon dissociation)[158,159]. This “hot-spot” region (major impact from Trp99 and Trp332) of the conformationally rigid Gβγ dimer is quite flexible allowing the variable binding of the unit with GPCRs (such as M1 and M2[160]), the Gα subunit, downstream effectors and peptides[161].

Even though modulation of GPCRs is not the main objective of the present thesis, structural features of the Gα-GPCR interface provide insightful knowledge not only for the canonical, but also on how to target non-canonical G protein-mediated signaling. Receptor-G protein complex structures have been resolved[48,126,127,162] pointing out that the N-terminus of this seven transmembrane (TM1-7) protein is located extracellularly, whereas the α-helix of the C-terminus acts as a bridge with the inner site of the cell[162,163]. The TMs are connected to the membrane with three ECL (ECL1-3, extracellular) and three ICL (ICL1-3, intracellular) loops[46]. The communication between the receptor and the respective G protein is established via different sites: the N-terminus, the α3-β5 and αN-β1 loop, and the domains α4 with the α4-β6 loop, and the α2-helix with the α2-β4 loop[46,164,165]. Upon conformational changes, the contact site between Gα and Gβ via the αN-helix and the β1-sheet can also facilitate contact between the receptor and the protein (Figure 7b)[166]. Since the nucleotide binding pocket is 30 Å away from the Gα- GPCR interface, only allosteric interactions would have an effect on the nucleotide pocket conformation. As a matter of fact, the GPCR-Gαβγ complex displaces the α5-helix of the Gα subunit upon activation of the receptor, which in turn leads to conformational changes of the β6-α5 loop (TCAT motif) and subsequently affects the nucleotide affinity[129,166]. Thus, the α5-helix plays a key role for the

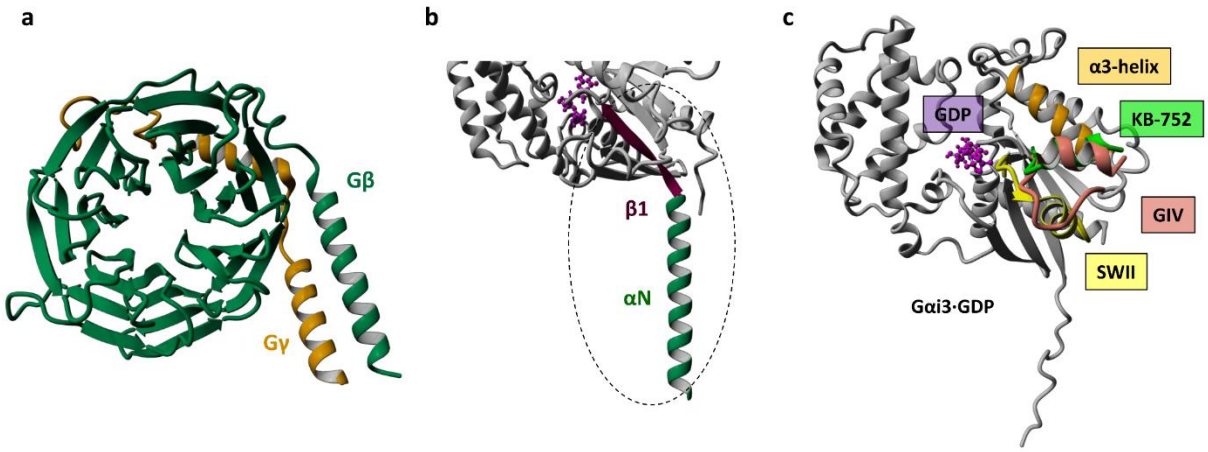

Figure 7: Structural depiction of significant G protein areas. (a) Crystal structure of the Gβγ unit (PDB ID: 1GP2[155]), where Gβ is shown in green and Gγ in orange. (b) Illustration of the β1-sheet and the αN-helix on Gαi1·GDP HM. (c) The SWII/α3 hydrophobic cleft in Gαi3·GDP. Superimposition of the existing crystal structures of Gαi3·GDP with two Gαi modulators, GIV (PDB ID: 6MHF[9] in pink) and the peptide KB-752 (PDB ID: 6MHE[9] in green).

receptor-protein connection. Another allosteric interaction involves the αN-β1 loop, which is getting more dynamic upon interaction with the receptor shifting the ICL2 closer to the P-loop via the β1-strand[127, 162,167]. Also, conformational changes of the SWII region have been reported to affect the GPCR-G protein affinity, especially for the Gαi and Gαs proteins[168,169].

Finally, a plethora of crystallographic studies supports the importance of the SWII/α3 region (Figure 7c) involved in the Gαi/s protein interaction with AC[44,51,123], with GEFs (GBAs; Gα-binding and -activating proteins[170,171], Ric-8[136,145,146,172]) and GDIs (GPR[32]), but also with synthetic compounds such as GIV-Girdin[9], KB-752[9,173,174], and GPM-1[21], as well as effectors (PDEγ[36]), as will be analyzed later (chapters 1.2.1 and 1.3). Therefore, this specific region has been tightly associated with the affinity for guanine nucleotide binding and utilized for G protein modulation.

1.2 Targeting Gαi/s proteins

In the previous chapter (1.1), the activation mechanism of heterotrimeric G proteins in the context of GPCR-dependent or -independent signaling has been discussed extensively. Due to the lack of adequate and accurate information on chemical, biological and structural properties of the G proteins, their broad application as pharmacological tools is restricted. To date, a great number of reviews has been published, summarizing the potential utility of G proteins as therapeutic targets upon their intracellular interaction partners[6,8,43,117,154,175]. The majority of these studies though claimed the difficulty of targeting directly G proteins, because of their intracellular localization[6,8,176]. Unlike GPCRs, molecules directly addressing G proteins in the cell interior, are not trivial to be delivered since the proteins do not possess any extracellular binding site. By assessing the druggability of Gαi/s proteins, the initial point would be to identify the different interfaces of their PPI sites, i.e., Gαi/s-GPCRs, Gαi/s-effectors, Gαi/s-Gβγ, Gαi/s-accessory proteins and Gαi/s-guanine nucleotide binding pocket. A step further would be to provide the sufficiently pure and active proteins as well as specific and potent ligands as modulators both produced and characterized with straightforward and efficient (bio)chemical, bioanalytical,

biophysical, and lastly, biological strategies. This subchapter outlines the known methodologies and discusses the current restrictions and possible alterations to pursue these tasks.

1.2.1 Strategies towards targeting Gαi/s proteins: scanning of protein-protein interaction sites to overcome inaccessibility

Motivated by the aforementioned obstacles, we compiled the available knowledge concerning the functional sites of the Gαi and Gαs protein subunits to get a deeper insight into the PPIs these proteins are involved in and to evaluate the different interfaces of the Gα monomer with associated interaction partners and artificial compounds, as far as available. The C-terminus of the Gα subunit has shown a relatively high specificity for the GPCR-G protein interaction[5,177]. Also, the N-terminus has been found to be addressed via cationic amphiphilic peptides, such as mastoparan[5,178,179]. This tetradecapeptide can adopt an amphiphilic α-helix structure[178]. In this way, the ligand has shown affinity for both Gα protein termini[177,178,180], increases the cell permeability due to the load of basic residues, and competes with the interaction between the receptor and the protein[178–180]. Thus, development of amphiphilic compounds targeting the dissociation of the GPCR-Gαi/s complex would be interesting to study the modulation of the respective protein signaling.

Advances in G protein-mediated signaling are primarily focused on Gαq[94,181,182], Gαi and Gαs, with the two latter ones being in the spotlight for the last two decades[22,49,59,78,100,183–187]. The converse influence of Gαi/s activation on the common effector, AC, and the subsequent second messenger, cAMP, inspired the research community to investigate the core function of these proteins both biologically and structurally. As already mentioned above, the AC enzyme comprises nine isoforms and the active site is located between the two cytosolic domains C1 and C2[188]. Studies revealed that the Gαs protein interacts with ACI-IX, whereas Gαi only with the three isoforms ACI, ACV, and ACVI. Post-translational modifications (PTMs), such as myristoylation of the Gα subunit[16,17,57,189], have been described to have an inhibitory effect on the G protein-AC interaction at a cellular basis. There are even computational works indicating that a non-myristoylated Gαi protein is structurally similar to Gαs and would not inhibit the AC activity[190,191]. Therefore, an interesting aspect would be the comparison of both Gα protein subfamilies with and without PTMs. In parallel, a widely used activator and subsequently intracellularly indicator of AC is the diterpene forskolin (Fsk, Figure 8a)[192,193]. This reagent is used as a “method-to-go” for different biological assays on membrane preparations, cells and tissues[21, 47,194–196]. Additionally, the Gαi/s protein-AC interaction can be further addressed and monitored via GPCRs, e.g., by applying isoproterenol (Iso, Figure 8b), AC is indirectly activated after the binding of Iso to the β_2-AR[197–199]. DAMGO (D-Ala^2-N-MePhe^4-Gly-ol^5]enkephalin[200,201]) and DADLE (Tyr-D-Ala^2-Gly-Phe-D-Leu^5]Enkephalin[202], Figure 8c) were reported to downregulate the intracellular cAMP level by activation of the μ and δ-opioid receptors, respectively. Although the above reagents are applied regularly for monitoring the G protein-mediated downstream signaling, the effector interface has not yet been drugged[5]. Due to the flexibility of the Gβγ dimer and its various interactions with accessory proteins (such as AGS or RGS) or considering the imbrication with the effector molecule binding site[158], there are only few examples of possible strategies targeting this site. As known, the SWII/α3 region is a

common interacting area accounting for the modulation of the Gαi/s proteins. The RGS14 GoLoco motif, for example, binds to the SWII/α3 region of the Gαi protein and interferes with the contact between the monomer and the dimer, hindering the heterotrimer re-association[85,89,203,204]. Apart from this molecule, suramin, which was initially a drug discovered by Bayer AG back in 1916, indicated binding close to the

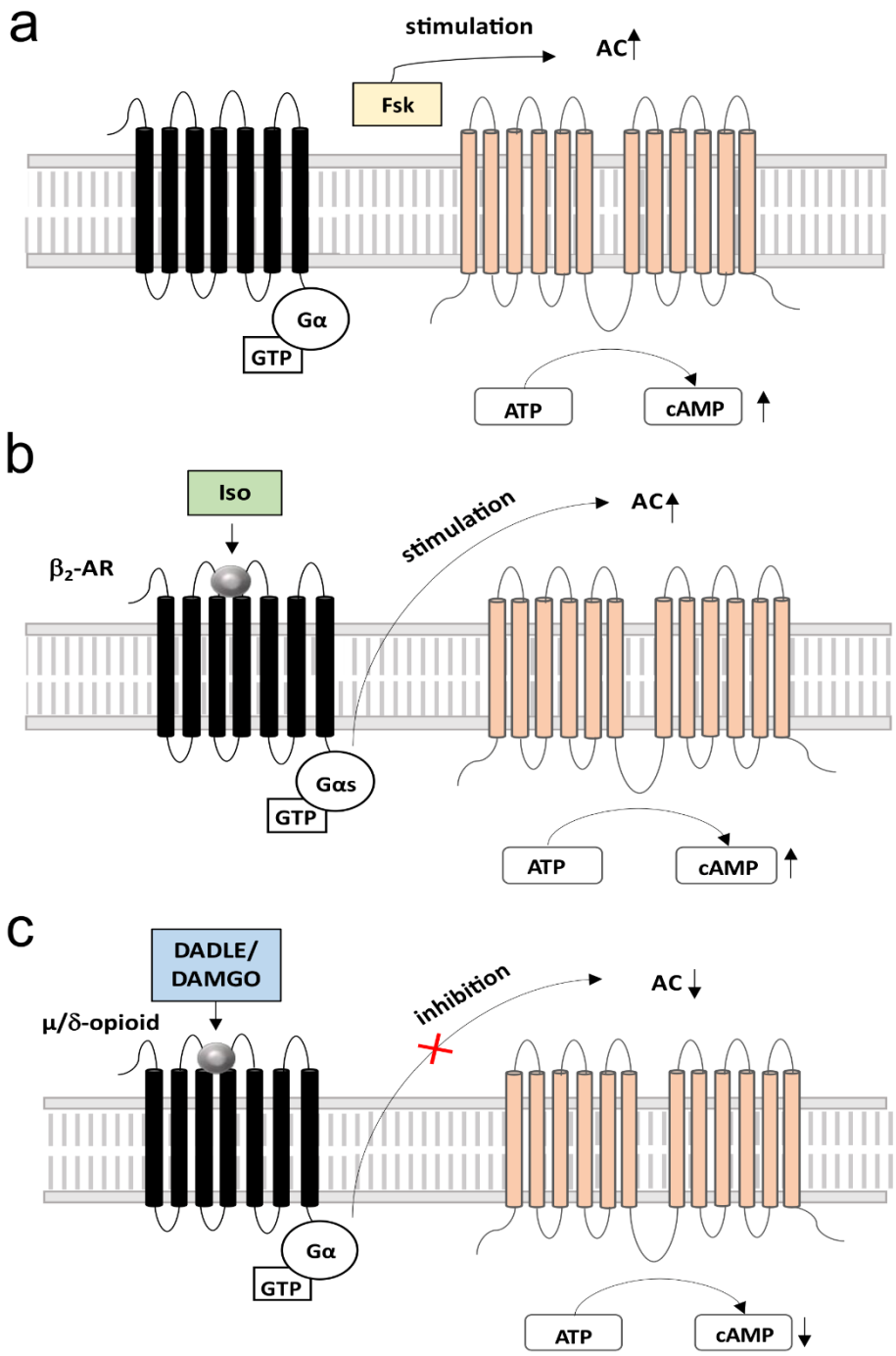

Figure 8: Modulation of effector signaling. (a) Schematic illustration of receptor-independent stimulation of adenylyl cyclase (AC) via forskolin (Fsk). Subsequently, intracellular cAMP levels are increasing. (b) Isoproterenol (Iso) binds to the β_2-adrenergic receptor (AR), activating the Gαs signaling cascade, which in turn stimulates AC and converts ATP into cAMP. (c) DADLE binds to the δ-opioid receptor, which subsequently inhibits the AC stimulation via Gαi proteins, thereby activating the Gαi signaling with subsequent intracellular cAMP downregulation. This figure is modified from Pepanian *et al.*[140].

Gα-Gβγ interface inhibiting both Gαi/s protein subunits thus lowering the selectivity over the targeted protein[205]. This binding site can overlap with the binding of surrounding accessory proteins, effectors[117], or receptors[206] and could lead to diminished selectivity.

Despite the tremendous efforts undertaken so far to tackle the druggability of the Gαi/s proteins, the most promising results and positive lead compounds are targeting the Gα-nucleotide and the Gα-accessory protein interface. Referring back to the GTPase cycle, the key feature concerning the protein state is the nature of the bound guanine nucleotide in the core of the Gα subunit. Although the nucleotide binding pocket is not a typical target-area, it can provide vital information regarding the interactions occurring between the ligand and the protein, which subsequently affect the nucleotide binding stability[30,207–209]. This can be further elaborated with the exploration of the different accessory proteins that come into the game[210]. The aforementioned categories of these assisting proteins, i.e. GEFs, GAPs, GDIs, and the recently discovered GEMs (guanine exchange modulators), distinguish their functions depending on their ability to promote or suppress the guanine nucleotide exchange[132]. From a further tool development aspect, one could focus on two individual, but closely related, views. Foremost, and as mentioned in the beginning of this section (1.2), the intrinsic GTPase activity needs to be evaluated. Already beginning in the 1980's, researchers were trying to observe and estimate the binding of the nucleotide to the Gαi/s proteins[180,207,211,212]. The discovery of guanine nucleotide analogs (fluorescently or radioactively labeled derivatives) has provided substantial knowledge as will be unraveled in the following sections (1.2.2 and 1.2.3)[50,207,208,213–215]. The second aspect to be considered includes the ability of accessory molecules to establish or break bonds formed between the respective (anta)agonist and the catalytical residues of the protein. Compounds that bind to, or close to SWII/α3 are regularly proposed by many studies since occupation of this area would mutually exclude the interaction with the Gβγ dimer[9,20,22,145,155]. In terms of GDP exchange into GTP and thus initiation of the G protein signaling, residues such as Thr35 and Gly60 are of interest since they interact with the Mg^{2+} ion and lead to conformations that facilitate the γ-phosphate binding[13,216]. Also members of the G-R-E triad (chapter 1.1.2) and their subsequent behavior would potentially decode the heterotrimer association[144]. On the other hand, interactions of Arg178 and Gln204 with residues that could attack GTP nucleophilically, can account for the GTP-hydrolysis mechanism resulting in GDP attachment and G protein inactivation (Figures 5, 6)[36]. Overall, many diverging PPI sites, residing on the Gα protein subunit, exist. The guanine nucleotide binding pocket along with the interaction site with accessory molecules display high prominence for the regulation of Gαi/s signaling and merit further examination.

1.2.2 Determination of the Gαi1 protein activity

Until 1999, three different subforms of Gαi (Gαi1, Gαi2, Gαi3) were discovered sharing a 85-93% sequence similarity (Figure 1)[29,217]. The first studies of characterizing the inhibitory Gαi protein subunit aimed at the investigation of regulatory components within the AC[30,218]. The protein was purified from rabbit liver membranes with a relatively low yield of 5% and was tested for nucleotide binding after ADP-ribosylation[30]. The studies revealed that the Gαi1 protein exhibits high affinity towards several guanine

nucleotide analogs in the following rank-order: GTPγS (guanosine 5'-O-[γ-thio]triphosphate) > GMPPNP (guanosine-5'-(β,γ-imido)triphosphate or Gpp(NH)p) = GTP = GDP > GMP (Figure 5)[30]. Binding of the natural nucleotides like GDP and GTP, but also of the non-hydrolyzable analog GTPγS was monitored via the intrinsic fluorescence of the G proteins. The changes of the fluorescence intensity of tryptophane residues upon protein and nucleotide binding can indirectly assess the active conformation[119,211,219]. However, this approach is not always proper due to low sensitivity[16]. Initial indications report the slow nucleotide association (approximately 60 min) via utilizing radiolabeled [^{35}S]GTPγS. In general, direct spectroscopic quantification of nucleotide binding is restricted due to a huge consumption of the studied protein[13]. An enhanced binding was verified with the addition of Mg^{2+} to the solution, as was expected for the exchange of GDP into the GTP analog. Aiming at the reduction of contamination of the protein fractions and the increase of the yield, recombinant Gαi protein purification was reconstituted in *Escherichia coli* (*E. coli*) bacteria cells instead of mammalian cells[214,220]. N-terminal modifications, such as myristoylation, ensure the membrane localization of the protein, whereas lipid-unmodified protein fractions derived from *E. coli* cells would justify the diminished affinity to the Gβγ dimer[221]. Since the 1990's[214,220–224] until today[16,17,125,134,145,146,225,226], the solubility, folding and activity of recombinant G proteins is put under the microscope. This can be applied for Gαi and Gαs (and their isoforms), since Gαq/11 and Gα12/13 subunits have been found to be difficult to be expressed in bacterial cells in a soluble and active form and therefore insect cells are preferred here[14,124,227,228]. The protein expression in prokaryotic systems is in general an inexpensive, non-demanding, and time-wise shorter approach for obtaining high amounts of the protein of interest[229,230]. Among all Gα protein subunits studied concerning recombinant protein production in bacterial expression system, Gαi resulted in the highest amount of soluble protein (50 – 400 mg pure Gαi1 in 10 l bacteria culture[125,220,225,226]). In most reports, the Gαi1 protein under study was tagged at the N-terminus (6xHis-tag, hexahistidine tag) to facilitate further purification steps. This terminus region is preferred since the C-terminus is known to be the binding site for the GPCRs[222,231]. A drawback of the above methodology is the relatively poor solubility obtained (50% recovered from cell lysates[223]). Yet, the Gαi subunit and especially the Gαi1 isoform, represents the most soluble and yield-wise efficient isoform compared to other Gα subfamilies[134, 220, 222,225]. Possible strategies to overcome the solubility issue or potential protein misfolding are refolding approaches (which were so far relatively futile[134,222,225]), fusion proteins (e.g., with receptors[232,233]), co-expression of accessory proteins (e.g., with chaperones[172,234]), or expression as chimeric protein forms[121,235–237]. There is no doubt that the production of high amounts of correctly folded, pure Gαi1 protein subunit is essential for the conduction of further biological and structural studies. Therefore, the recombinant protein needs to be first assessed for its function. As referred in the beginning of this section, stating that the nucleotide binding to the Gα subunit is the key point in the process. The majority of the studies exploited the non-hydrolyzable, radiolabeled [^{35}S]GTPγS nucleotide in order to quantify the GTPγS binding affinity. Despite the fact that this analog is still utilized for measurements, it tends more and more to be replaced with more with more environmentally friendly substitutes, such as the fluorescently labeled analogs, BODIPY FL GTPγS (guanosine 5'-O-(γ-thiotriphosphate) N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacine-3-yl) methyl) thioester), (2'/3'-O-(N-methyl-anthraniloyl)-

guanosine-5'-(γ-thiotriphosphate) MANT-GTPγS, and (2'/3'-O-(N-methyl-anthraniloyl)-guanosine-5'-(β,γ-imido)triphosphate) MANT-GMPPNP[16,17,125,213,215,225,238,239]. For direct observation and determination of BODIPY/MANT-nucleotide binding to Gαi1, the protein was initially myristoylated and bacterially expressed due to further reconstitution in lipid vesicles[16,17,207,209], and dissociation constants were determined via fluorescence measurements. For both nucleotide analogs, GTPγS ($K_D \approx$ 75 – 150 nM) exhibited a higher binding affinity compared to its GMPPNP counterparts ($K_D \approx 1.5$ μM)[17,209].

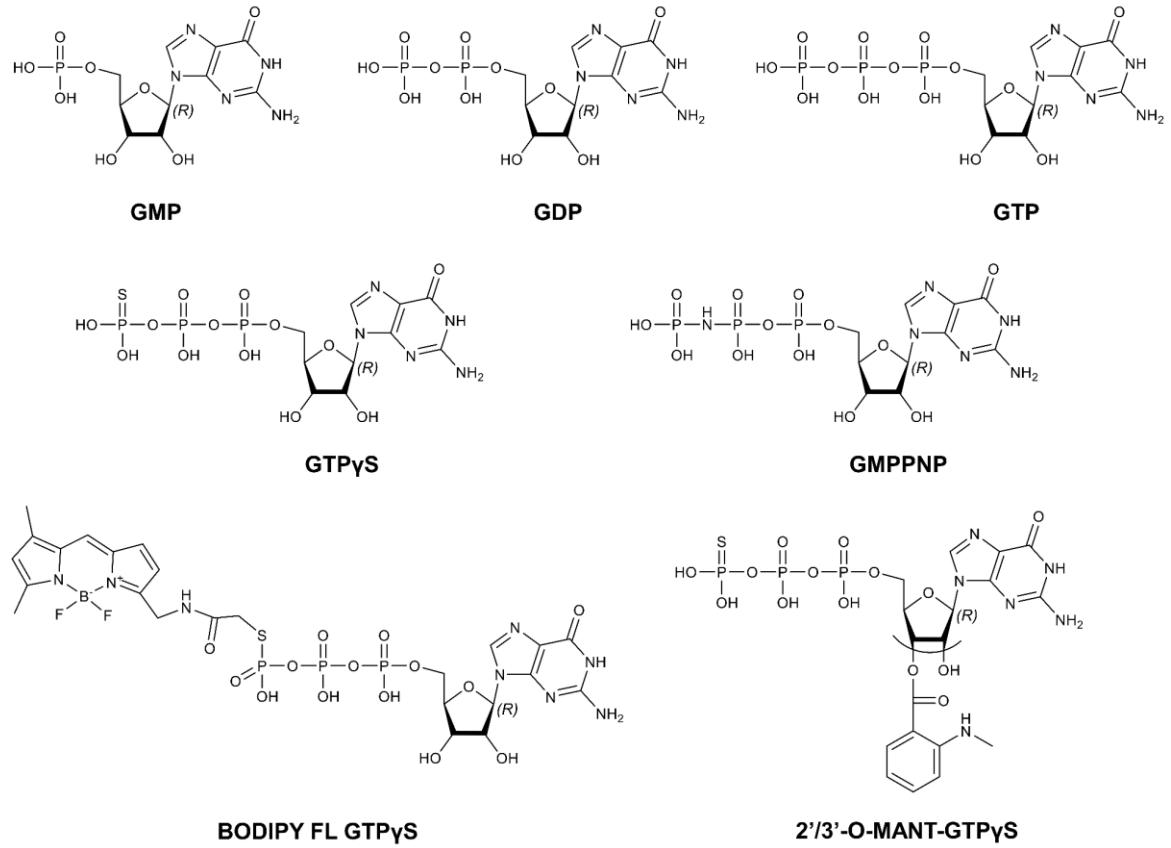

Figure 9: Chemical structures of different guanine nucleotide analogs. The full names of each compound are described within the text (chapter 1.2.2).

Apart from the ecological pre-eminence of the above compounds compared to radiolabeled nucleotides, they facilitate the real-time observation of nucleotide binding. The spectral properties of the BODIPY FL-fluorophore (higher fluorescence intensity[16]) are more favorable in contrast to the requirement of the MANT-probe for wavelength of 280 – 290 nm, which would require distinct optical components. Although the broad use of BODIPY FL GTPγS for monitoring the ability of Gαi to be activated is unquestionable, probes with similar spectral features that could bind to the Gα subunits with higher binding affinity (low nM range) and specificity would provide quantitative information as desired.

1.2.3 Determination of the Gαs protein activity

A similar concept was also followed for the Gαs protein subunit, which exhibits a ~40% sequence similarity to the inhibitory G protein (Figure 1)[29]. Three exon junctions of Gαs and Gαi are conserved,

implying their common ancestral origin[240]. Two main isoforms of Gαs gene (Gαs(long) and Gαs(short)) are expressed ubiquitously in all tissues in humans[141] and mutations that up- or downregulate their function can lead to tumors, iPPSD (inactivating PTH/PTHrP signaling disorder) or McCune-Albright syndromes[53,241]. Their molecular weight was initially described to be 45 – 52 kDa, with the broader band being electrophoretically detected as a doublet[242]. Depending on the tissue-requirements, cells express diverse ratios of the Gαs(long) and Gαs(short) subunits. For instance, the long isoform is abundant in the kidney[243] and placenta[244], whereas the short is found in the heart[245] and platelets[246]. However, cDNA isolation from bovine/rat brains indicated splicing of the mRNA at the exon 3 position, accounting

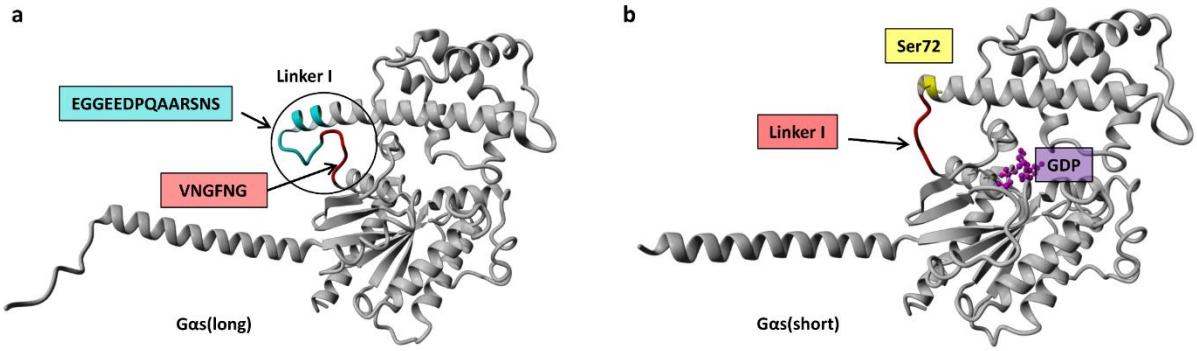

Figure 10: Structural comparison between Gαs(long) and Gαs(short) isoforms. (a) Structural depiction of Gαs(long) (UniProt: P63092-1). In the circle, the linker I region of the protein with the additional amino sequence illustrated in cyan and the common area with Gαs(short) is shown in red. (b) Crystal structure of Gαs(short)·GDP (PDB ID: 6EG8[48]), highlighting in yellow the substituted Ser72, which can further be phosphorylated[242].

for the heterogeneity between the Gαs variants[217,242,247]. The two spliced variants, share high sequence similarity differing primarily in their AHD region[135,248]. The GNAS2 gene encodes a sequentially 14 amino acid shorter protein (absence of 42 nucleotides, Figure10a), hence the name Gαs(short), with two additional amino acid replacements. Glutamate (in the long version) is replaced by aspartate at position 71 of the Gαs(short) isoform and glycine (in the long form) into serine (in Gαs(short)) at position 72 (Figure 10b)[242]. The addition of serine could enable the subsequent serine phosphorylation by PKC[240,249]. Crystallographic studies of Gαs(short)·GTPγS (PDB ID: 1AZT[44]) provided structural information on critical residues. The additional stretch of the long isoform, which is normally in close proximity to the linker I region, was not detected, presumably due to high flexibility.

Analogously to the Gαi1 subunit, attempts to express the Gαs subunit in *E. coli* were also performed[212, 220,222,224,250]. Apart from select works on both homologs[224], in most cases of Gαs expression in either eukaryotic[251–253] or prokaryotic[212,220,222,250] cells, the short variant is produced. An overview of the expression systems and corresponding conditions used for the production of Gαs(short), Gαs(long), and Gαi1 subunits is listed in Table 1. The protein production still lacks efficacy due to the solubility issue and, subsequently, yield. There are even studies stating that Gαs(short) expression in the soluble form is not feasible due to instant protein aggregation in the pellets forming inclusion bodies[225,254]. Along with it, it is reported that a high proportion of the purified protein (30-90%) remains inactive[255]. Evidently, the resulting amount of the purified Gαs(short) is much lower compared to Gαi1, with only 1 – 35 mg pure

protein being retrieved from a 10 L bacteria culture approach[212,220,224,225,250]. Differences in the expression system did not significantly alter the outcome[51,135,162]. Following a similar pattern as for Gαi1, the refolding of the Gαs(short) protein was not successful to recover properly folded protein[225]. The affinity of the protein for nucleotide binding was facilitated as before with [^{35}S]GTPγS nucleotides, indicating that Gαs binds to GTPγS with lower affinity but in a faster manner (30 min.[135]) compared to the Gαi protein[30].

Moreover, Graziano *et al*.[248] stepped further, showing that the long isoform binds less GDP than the short, which was comparable to the behavior of the receptor (β_2-AR)-fused Gαs. Addition of Mg^{2+} ions also enhanced the nucleotide binding, with the elevated amounts of Mg^{2+} being required especially for the GTP hydrolysis step[36,216]. A total magnesium content of ~20 mM within the organelles has been estimated, with ~5 mM Mg^{2+} corresponding to the free amount in the cytoplasm of mammalian cells[256]. Apart from the slight differences regarding the reaction kinetics observed between the two isoforms, no distinct outcome with respect to the functional discrepancies has been reported. Increased, unchanged, and also reduced levels of AC were reported upon activation of both Gαs isoforms[186]. These observations highlight the controversy considering the function of the Gαs isoforms with an accurate explanation still missing. Due to the fact that the AHD is usually described as a very dynamic and flexible region[44,135], and the additional 14 amino acid sequence is located close to the linker I region of the AHD, the vast majority of experimental and structural studies are applied using the Gαs(short) isoform, which is still ongoging[17,22,123,126,128,146,162,225,257,258].

In the context of activity determination, initial indirect approaches, such as the trypsin protection assay[15,225] were carried out, and the broadly-used fluorescently-labeled nucleotide analogs, BODIPY FL GTPγS[16,17,50,241], MANT-GTPγS and MANT-GMPPNP[49,50] were utilized. Studies of human[129,225,259–262] or bovine[44,51 224,247,250,263] Gαs(short) with[17,222,264] or without (in lower extent) modifications, have been successfully done and provided important information about the protein-receptor (β_2-AR[48–50,126,128, 162,265]), protein-effector (AC[44,47,51,53,149]) or protein-accessory protein (Ric-8[146,172,263,266]) interactions. Radioactivity studies indicated a binding of ~0.6 – 0.7 mol GTPγS/ mol Gαs(short). Dissociation constants are available only for the complex of the myristoylated, myrGαs(short)-BODIPY FL GTPγS, i.e., $K_D \approx 70$ nM. The MANT-based probes were found inadequate to activate Gαs(short) proteins[50]. Purified Gαs proteins are conformationally more flexible than the membrane proteins, therefore binding of bulky nucleotides can lead to variable binding affinities and its fusion with additional molecules, such as a receptor or AC, is preferred[49,50]. Therefore, future studies exploring deeper the functional differences between the two isoforms of Gαs, providing information on protein activity and structural conformation would be of paramount importance. Chapters 3 and 4 describe the contribution of this thesis to the investigation and determination of the activity of non-modified, human recombinant Gαi1, Gαs(long), and Gαs(short) protein subunits.

Expression system	Protein subunit	Expressing vector	Organism	Ref.
***E. coli* cells**	Gαs(short)/(long)	K38 strain, pT7-5	bovine	Graziano *et al.*[224]
				Graziano *et al.*[212]
	Gαs(short)	BL21 (DE3) strain, NpT7-5		Itoh *et al.*[250]
		BL21 (DE3) strain, pET28a		Sunahara *et al.*[44]
				Du *et al.*[126]
		BL21 (DE3) strain, pQE-6		Taussig *et al.*[57]
				Nagai *et al.*[263]
		BL21 (DE3) strain, pREP4		Sunahara *et al.*[188]
		BL21 (DE3) strain, pET15b	human	McCusker *et al.*[225]
	Gαi1/13 chimera	JM109 (DE3) strain, pET28a		Chen *et al.*[236]
	Gαs(short) & Gαi	BL21 (DE3) strain, pET28a		Dror *et al.*[129]
	Gαs & Gαi	BL21 (DE3) strain, pQE-6	n.a.	Lee *et al.*[220]
		BL21 (DE3) strain, pET21a		Srivastava *et al.*[341]
High5 cells	Gαs(short)	AcNPV	bovine	Chung *et al.*[265]
	Gαsβγ	pVL1392, BestBac	human	Zhang *et al.*[127]
	Gαs(short)	BestBac		Liang *et al.*[130]
				Gregorio *et al.*[259]
		pFastBac	bovine	Qi *et al.*[51]
		AcNPV		Rasmussen *et al.*[162]
	Gαi/q chimera	n.a	n.a	Tesmer *et al.*[151]
Sf9 cells	Gαs(long) & Gαi1	pcDNA3.1+, pFL/pIDC	human	Hillenbrand *et al.*[251]
	Gαs	pBN-181, AcNPV	n.a	Markby *et al.*[253]
	Gαs(short)βγ	BacPac6 viral DNA	human	Yao *et al.*[252]
	Gαi1	n.a	n.a	Kozasa *et al.*[343]
	Gαs(long) & Gαi	pVL1393	bovine	Graber *et al.*[264]

Table 1: Overview of existing expression systems applied for human or bovine Gαs(short), Gαs(long) and/or Gαi protein subunits. n.a: not available.

1.3 Modulation of the Gαi/s protein subunit

The conditions and limitations of the Gαi/s proteins being intrinsically activated gave rise to the discovery of natural or artificial compounds that can modulate the Gαi/s-mediated signaling. These molecules usually target and bind the Gα subunit either on the GTPase domain or on the interface between the two domains, close to the nucleotide-binding pocket. The established PPIs cause conformational changes on the SWI-III regions thus enabling or inhibiting the nucleotide exchange.

The most commonly used activator of the Gαs subunit is the exotoxin secreted from *Vibrio cholerae*, *cholera toxin* (CTX)[267]. CTX induces ADP-ribosylation of the catalytical Arg201 of Gαs, permitting permanent GTP binding through GTPase activity inhibition. This leads to perpetual AC and subsequently cAMP production, closely related to cholera disease[268]. On the other hand, suramin, the molecule that was previously introduced (chapter 1.2.1), acts as a blocker of Gα protein function due to the inhibition of the GDP-GTP exchange, which results from suppression of the GDP release on the Gαs subunit. Unfortunately, the compound is neither cell permeable (because it is negatively charged) nor highly selective, since it shows affinity for both Gαs and Gαi[205,206]. These features restrict its broader pharmacological application[8,269].

Another toxin, i.e., *pertussis toxin* (PTX), produced from *Bordetella pertussis*, is able to ADP-ribosylate G proteins comparably to CTX. It belongs to the family of A-B toxins, with the A-oligomer part being Gαi-dependent (ADP-ribosylation of the Gαi protein), whereas the B-segment is associated with cell-surface proteins (Gαi-independent)[270]. This ADP-ribosylation is facilitated through Cys347 in the Gαi subunit and leads to inhibition of the Gαi signaling pathway (GDP-bound state). Although PTX is also widely used to monitor Gαi signaling, it acts irreversibly on the protein[271].

The breakthrough discovery of a novel and, in mammalian tissues, widely expressed protein is the G-interacting vesicle-associated (GIV or Girdin) protein[272]. The interaction site between GIV and Gαi/s protein was initially found to be facilitated by 83 highly charged and conserved amino acids[272]. A 20mer GBA motif was discovered at the C-terminus of the GIV protein (i.e. amino acids 1674–1694[20,273]) and first described to act as a non-receptor GEF (chapter 1.1.1). Also, DAPLE, CALNUC and NUCB2 are homologs that were found to belong to the same family as GIV/Girdin[171]. This GBA motif of the GIV protein was found to be essential for non-canonical G protein activation in cells by detecting increased levels of Gβγ and Akt (through the Gβγ-PI3K pathway)[20,274]. Remarkably, GIV/Girdin is the prototypical protein described to possess a GEF activity for the Gαi subunit enhancing cell migration and, at the same time, a GDI activity that prevents Gαs-mediated signaling at the endosomes restricting further cell proliferation[80,275]. This bifunctional activity of a single short protein stretch has been defined as a whole new modulator category, the so-called GEMs (chapter 1.2.1)[80]. Although the GIV protein was discovered by yeast-two-hybrid assay the GEM-like activity of the molecule was determined based on its homology to a synthetic peptide, KB-752. The significance and contribution of peptides to G protein modulation is described in detail in the following chapter (1.4). Crystallographic experiments revealed that the binding site of GIV lies on the notorious SWII/α3 hydrophobic cleft and the α3-β5 loop (Figure 7c)[9]. Compared

to the GPCR-dependent G protein signal transduction, the non-canonical mechanism via the cytosolic GIV is prolonged and thereby the cAMP inhibition is lingering[101]. Considering all aforementioned aspects, the GIV protein family, and the discovery of compounds with a GEM-like activity could be the initial impulse for novel, potent, and selective Gαi/s modulators.

1.4 Peptides as G protein modulators

The application of synthetic peptides as chemical probes and pharmacological tools with therapeutic potential has been developed over the years[276–278]. In principle, small molecules have been widely used as therapeutics used to treat inflammatory or metabolic diseases[279–281]. Although they have favorable pharmacokinetic properties, they lack selectivity and specificity due to several, sometimes toxic, side effects[279,282]. Peptides and peptide constructs with other moieties are preferred for PPI modulation, however, their development is more challenging compared to small molecules[283]. At the early stage of research, where specific segments of proteins were identified to possess biological activity, synthetic bioactive peptides of the desired fragments (linear and macrocyclic) were produced and introduced in multiple studies in order to obtain a deeper insight into their mechanism of action[184,284]. Typically, bioactive peptides are between two to twenty amino acids long[285], but also longer examples exist. Among the first therapeutic peptides reported are insulin and oxytocin[280,284]. Two critical points for peptides to be considered as drugs, are the cell permeability for intracellular efficiency and stability against proteolytic degradation. A myriad of studies[283,286–290] highlights the greater efficacy of structural modifications of linear peptides for improved biological activity and physicochemical properties with only few exceptions indicating a similar effect[291]. Suggested modifications include (macro)cyclization, introduction of non-proteinogenic and/or D-amino acids, and conjugation of additional short CPP (cell-penetrating peptide) motifs, which reflect to enhanced structural constraint and subsequently higher binding affinity to the protein target and evolved cell permeability, respectively[286,289,290,292–294]. More specifically, the induction of unnatural amino acid residues could potentially increase the metabolic stability of the compound[295,296]. Typically, CPPs are polybasic (rich in Arg and Lys residues) and/or hydrophobic sequences, penetrating the cell membrane via endocytosis and being released from endosomes. Penetratin, R9 and TAT peptides are the most common CPPs used to test ligand cell permeability in drug development[297]. Introduction of CPPs does not only lead to molecules penetrating the cell membrane, but also provide proteolytic stability without cytotoxic side effects[298]. Studies modifying and, basically, optimizing the activity of CPPs by concomitantly getting vital information on the mechanism of endosomal escape (or internalization) were undertaken in recent years[298,299]. Thus, peptides also turned out to be promising molecular tools for modulating the “fate” of Gαi/s-mediated signaling. Medicinal chemical alterations and structural intervention of the ligands can alleviate significant difficulties, such as poor cell permeability and oral bioavailability. In the following chapters, the development of such peptides (chapter 1.4.1) and the required features (chapter 1.4.2) are thoroughly described.

1.4.1 Combinatorial peptide library screening

There are different methods available for investigating and developing peptidic scaffolds as biochemical and pharmacological tools. Combinatorial peptide library screening is in the front line for many years now, since more than 10^{14} members can be tested simultaneously[300]. The combinatorial peptide libraries provide remarkable diversity and a multitude of potential candidates. They are classified into the i) biological peptide libraries and ii) chemical libraries (Figure 11)[301,302]. To the first category belongs the DNA-encoded libraries, mRNA[303] or ribosome[304] display, which provide high diversity of scaffolds with the ability to include desired traits via mutations. The bottleneck of the latter approaches though is the mRNA lability[301]. A similar but cellular approach is phage display, which is the only library that results in mammalian-cell-binding protein isolation and allows DNA delivery to the host cells[302,305]. All above library

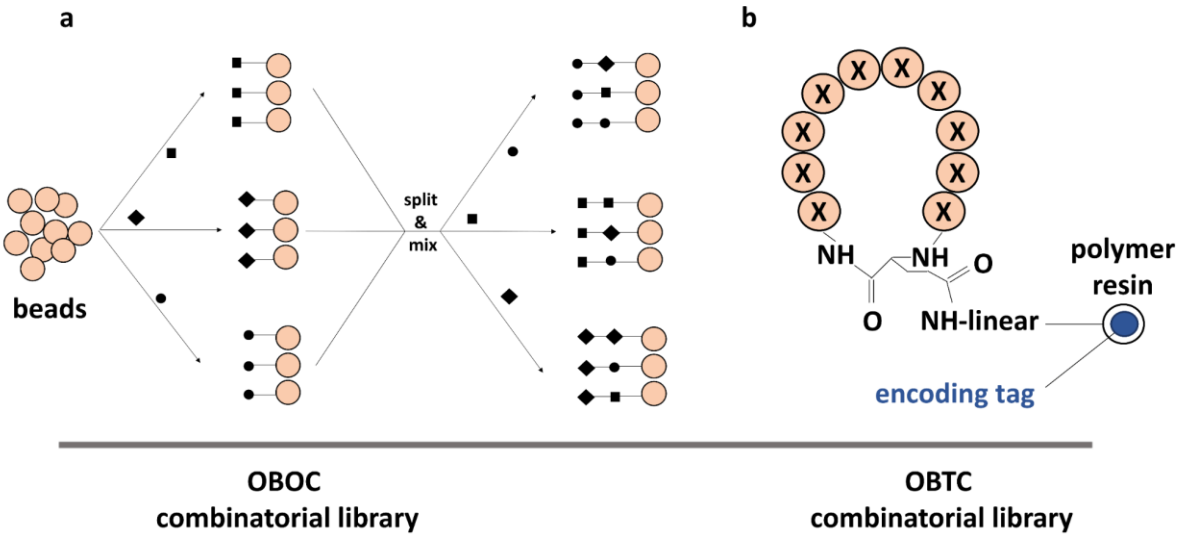

Figure 11: Chemical combinatorial peptide libraries. a) one-bead-one compound (OBOC)[302,306,307] and b) one-bead-two compound (OBTC)[18,24]. The figure was inspired by the references[24,308,309].

strategies arrange a huge assortment of peptidic scaffolds, but they are limited to natural and L-configurated amino acids only and therefore, neither structural nor cell-permeable properties are examined[310]. A typical combinatorial peptide library screening approach, the one-bead-one compound (OBOC) libraries, was introduced by Lam *et al*.[311] in 1991 (based on the concept described first by Furka *et al*.[312]) and belongs to the second group of the chemically-derived libraries. In the applied "split-and-mix" method of the OBOC library (10^6-10^8 library size[302]), each bead (80 – 100 µm) displays 10^{13} copies of the same chemical entity on its surface. The method has frequently been used in the past for ligand development targeting receptors involved in different cancer types[310] and in the elucidation of consensus sequences of protein domains involved in PPIs[18,290,313]. The target proteins, specifically Gαi/s, studied in the present dissertation, were also used for combinatorial peptide library screenings and therefore labeled with a tag (i.e., biotin[24]), which facilitates the detection of high-affinity binders as described in the following steps. The peptides are synthesized on a TentaGel resin and screened while still bound to the solid phase. The hit identification is performed via a colorimetric reaction catalyzed by alkaline

phosphatase, which is conjugated with streptavidin, and the substrate bromochloroindolyl phosphate (BCIP)[314,315]. The biotin-labeled protein is incubated with the OBOC library and after multiple washing steps (removal of unbound protein) and incubation with SA-alkaline phosphatase the addition of the substrate converts the protein-bound beads turquoise upon detection of a binding event, i.e., the presence of a peptide-protein complex on the beads. Positive hits are analyzed for their sequence with Partial Edman degradation (PED) and MALDI mass spectrometry[316–318]. In this way, the sequences can be identified, re-synthesized and tested for specific binding and activity towards the target proteins. Further chemical modifications, such as cyclization or the introduction of non-proteinogenic amino acids, as mentioned above, are possible within this library, however, may require adjusted chemical protocols. Kumaresan *et al*.[306] improved the existing OBOC strategy by introducing a known cell-capturing ligand on each bead surface. Within the same "split-and-mix" concept, one bead displays simultaneously a known ligand, and a random library compound, hence the name of the new library, one-bead-two compound (OBTC). Dehua Pei and his colleagues exploited the diversity and advantageous traits of both OBOC and OBTC libraries and applied them for combinatorial peptide library screening using different proteins, among them the small GTPase K-Ras[23,309,319].

1.4.2 Linear and macrocyclic peptides for Gαi/s protein subunits

Two outstanding paradigms of peptidyl Gα modulators are two cyclic depsipeptides, YM-254890 (YM) and FR900359 (FR). Both were discovered as natural products from bacteria and have shown an inhibitory and highly selective function towards the Gαq protein subunit. The YM depsipeptide, isolated from the bacteria *Chromobacterium* sp. QS3666[320], binds between the two interdomain linkers of the AHD and GTPase-domain of the Gαq subunit[321]. Additionally, the FR compound was extracted from the bacterium *Candidatus* Burkholderia crenata occurring as symbiont of the plant *Ardisia crenata sims*[322] and exhibited a similar GDI-like activity for the Gαq/11 protein subfamily[182,323]. Both cyclic depsipeptides have been chemically modified resulting in various analogs[182], none of which though could exhibit the similar potency and selectivity of the lead compounds. However, their structure gave impetus for further research and application of linear and macrocyclic peptides for the functional modulation of the Gαi/s subfamilies.

The combinatorial peptide library screening methods mentioned above resulted in diverse peptide scaffolds, including linear and (macro)cyclic peptides with or without unnatural amino acid residues. The most common strategy for peptide synthesis is the solid-phase peptide synthesis (SPPS). The pioneering development of SPPS was conducted by Bruce Merrifield (Nobel Prize in 1984[324]) in the 1960s and it is currently widely used e.g., in different approaches, including the synthesis of peptides and biopolymers, combinatorial solid-phase chemistry and chemical ligation strategies[325]. Starting with the linear modulators of Gαi/s subunit, peptides R6A(-1)[174,326,327], AR6-05[328] and GSP[184] from mRNA display, and KB-752 peptide from a phage-display study[11,12,174] were discovered. Remarkably, among all these peptides, only KB-752 showed a relatively high binding affinity (K_D = 3.9 μM[12]) for both Gαi1·GDP and Gαs·GDP by binding to the SWII region of the respective protein. This binding ability was also combined with biological activity revealing that KB-752 acts as a GEF for Gαi1 (inhibitor) and GDI

for Gαs (inhibitor)[11]. Previously, it was referred to the similar behavior noticed for the GIV/Girdin protein, where this dual activity was described as a GEM function. As a matter of fact, structural investigations of Gαi1·GDP with KB-752 and GIV indicated a common functional area (i.e. SWII-α3) and a shared GIV-GEM motif (ca. seven amino acid long), ΦTΦX[D/E]FΦ, where Φ: hydrophobic amino acid [21,80,170,329].

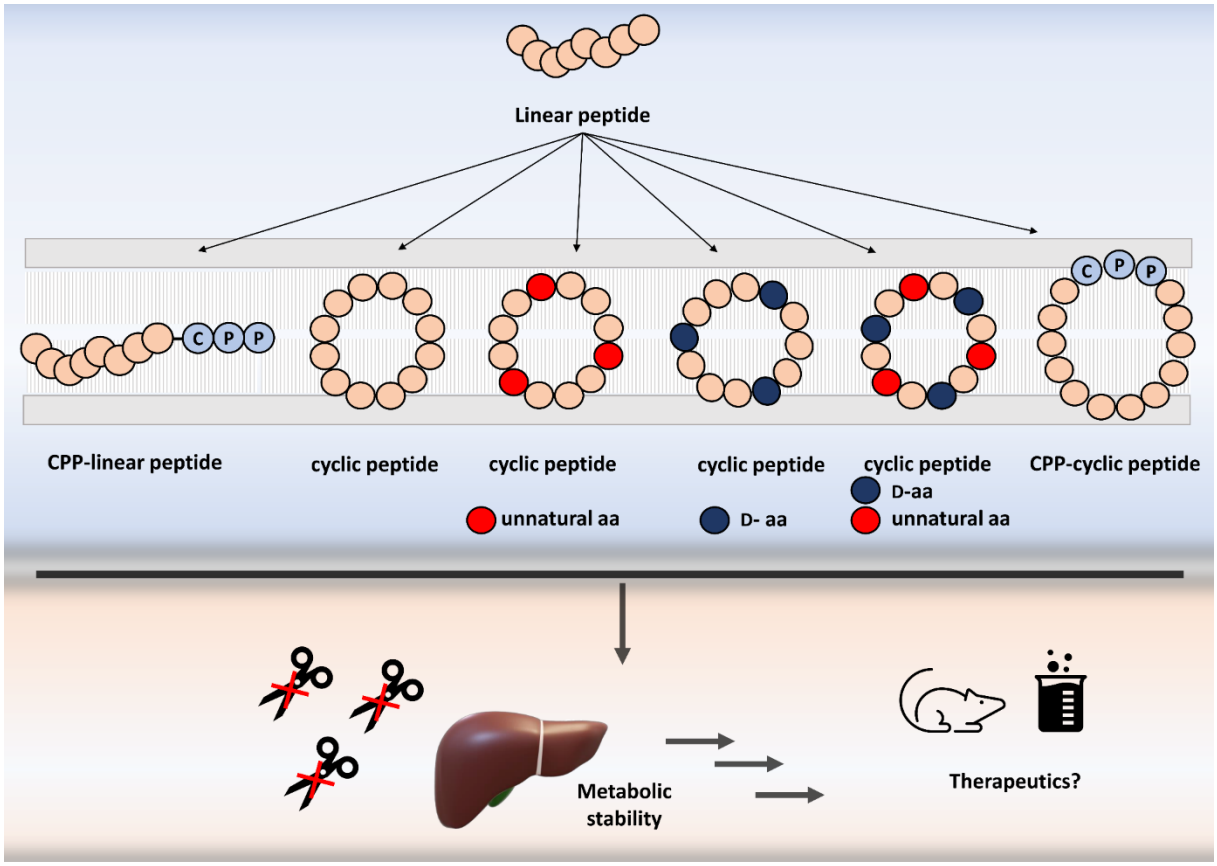

Figure 12: Schematic illustration of chemical modification of linear peptides to enhance cell permeability and metabolic stability[294,295,310,330]. CPP: cell-penetrating peptide, aa: amino acid, D-aa: D-amino acids.

With regard to the OBOC combinatorial peptide library approach, no linear peptidyl Gαi/s modulator possessing striking biological activity was obtained to date, whereas the OBTC was more fruitful at least for bicyclic peptides targeting the small GTPases[18,19,331]. The OBTC library screening approach was applied for inhibitor development of the monomeric K-Ras protein. Thereby, different so-called cyclorasins, such as cyclorasin B3[331], B4-27[18],and 9A5[23] have been discovered and characterized to possess low cytotoxicity, high metabolic stability and cellular activity for the Ras protein family. The positive pharmacokinetic properties resulted from the introduction of unnatural and multiple basic amino acid residues[295–297,310]. Three additional compounds derived from mRNA display by Richard W. Roberts and coworkers, namely cycGiBP[332], cycPRP-1[333], cycPRP-3[333], and Gα SUPR peptide[296] were developed for the Gαi1·GDP protein in the past. Unfortunately, therein no computational, and structural analyses regarding these compounds have been accomplished, which keeps information concerning the binding sites of the compounds and the target proteins still uncovered. In addition, two macrocyclic peptides, GN13 and GD20 were recently described for both states of Gαs protein subunits, derived from

a RaPID (random nonstandard peptide integrated discovery) system[22]. However, it is unclear whether the resulting hits are not only Gαs class selective, but also isoform (short and long) selective. The peptides exhibited a cellular activity upon addition of 25 µM peptide, a concentration that is relatively high and may deter from considering it as a suitable drug candidate, especially with an IC_{50} value of 12.21 µM. Taken together these factors and challenges, macrocyclic or bicyclic peptides still seem to be highly prominent for Gαi/s modulation, since monomeric G proteins resemble the GTPase domain of known small GTPases like K-Ras. Therefore, further OBOC and OBTC combinatorial library screenings could lead to efficient Gαi/s modulator development. Cellular, pharmacological, and structural investigations could be of paramount importance to address and solve the druggability of G proteins independent from GPCRs.

In the following chapters 5 and 6, it is referred in detail to the contribution to Gαi/s modulator development revealing three novel compounds, namely GPM-1, GPM-2, and GPM-3 with interesting biological activities. First, the linear peptide GPM-1 along with its derivatives, GPM-1b-d, were obtained from an OBOC library screening and indicated a GEM-like activity for the inactive state of Gαi1 and Gαs protein subunits. In the second peptide series, a OBTC peptide library was screened, and, as a result, two bicyclic peptides, i.e., GPM-2 and GPM-3 were identified that showed a potential GEF-like activity for Gαi1·GDP (GPM-2) and a GAP-like activity for the Gαi1 active state.

Details on the combinatorial peptide library screening as well as on the synthesis and characterization of the lead compound GPM-1 and its derivatives (GPM-1b, GPM-1c, and GPM-1d) can be found as preliminary work in the dissertation of Dr. Britta Nubbemeyer[334].

2. Strategies towards Targeting Gαi/s Proteins: Scanning of Protein-Protein Interaction Sites To Overcome Inaccessibility

Review article

Authors: Britta Nubbemeyer, **Anna Pepanian**, Ajay Abisheck Paul George, and Diana Imhof

This peer-reviewed article was published in *ChemMedChem*.

Citation: *ChemMedChem* (**2021**) 16(11), 1697–1716, doi: 10.1002/cmdc.202100039.

2.1 Introduction

Targeting G protein-coupled receptors in order to develop pharmacological tools is under study for several decades. To date, there are more than 30% FDA-approved drugs addressing these seven transmembrane receptors since their druggable sites are easily accessible on the cell surface[335]. Mutations and abnormalities in the GPCR-mediated signaling have been described in several pathological conditions[56,336]. Since there are close to 1000 different GPCRs[7,337], developing potent and selective drugs for GPCR is not a trivial goal, however, it has proven feasible by e.g., applying different types of antibodies and also small molecules in select cases. Out of 134 approved GPCRs-drugs, 61 are targeting Gαi/oPCRs and 40 GαsPCRs[4], whereas no G protein-targeting drug has been tested or approved so far[8,116,117]. Thus, we focused on investigating strategies that directly target the G protein-mediated signaling pathways. Irregular modulation of G proteins or alterations in the intracellular signaling cascades are involved in various diseases, such as heart failure and cancer[6,8,116,117,181,336]. The assessment of the different interfaces of Gαi/s proteins upon interaction with their effector molecules in the environment would enhance the druggability of these sites[175].

2.2 Summary and Outlook

Heterotrimeric G proteins are alluring pharmacological targets due to their topology and structure. They are membrane-bound proteins, which, in response to an activated-GPCR, undergo specific conformational rearrangements of their heterotrimer complex leading to dissociation of its subunits and subsequently stimulation of the respective intracellular signaling cascades[1,6,25,28]. Therefore, their vital role in transmitting extracellular information to the interior of a cell, through protein-protein interactions, is undoubtful[40,45]. In order to overcome the accessibility restrictions due to the intracellular localization of the G protein, this review summarizes and rank-orders the important determinants between the Gαi/s-GPCR, Gαi/s-G, Gαi/s-effector, Gαi/s-nucleotide, and Gαi/s-accessory protein interfaces[5]. For approximately 40 years (1980-today), exceptional studies discovered compounds, both natural and synthetic, that function as Gαi/s protein modulators, with more and more recent works unravelling and suggesting the GPCR-independent signaling modulation[6,7,10,30,62–65], since targeting selectively G proteins in the presence of GPCRs is not guaranteed.

With our review we also highlight structural regions and properties of the respective Gα protein subunit. The overlapping interfaces of Gαi/s-Gβγ and Gαi/s-effector with the accessory protein interface, renders the preference of targeting the first two interfaces. Among those interaction sites, the most promising interfaces were found to be the Gαi/s-nucleotide binding pocket and the Gαi/s-accessory protein interface. The nucleotide binding pocket constitutes the core of G protein activity regulation. The characteristic dual feature of the Gα subunit to first exchange GDP for GTP in order to be activated, but also intrinsically hydrolyze the bound GTP into GDP terminating the signaling is tantalizing for tool development. Literature screening revealed linear (KB-752[11], GIV-Girdin[9,20], R6A[174] etc.) and macrocyclic peptide candidates (cycPRP-1/2 and cycGiBP[332,333], GsNI-1[338] etc.) acting as accessory proteins, such as GEFs (guanine nucleotide exchange factors), GDIs (GDP dissociation inhibitors) or even GEMs (guanine nucleotide exchange modulators, see chapters 1.1.1 and 1.4.2)[9,11,20,32,80,174,184,339]. It needs to be emphasized that, at the time point of this review publication (2021), the work of Dai *et al*.[338] describing the GsNI-1 bicyclic peptide was stored in bioaRxiv and since 2022, it has been renamed to GN13[22]. For the Gαi/s-accessory protein interfaces, the main representative proteins are the so called RGS (regulators of G protein signaling), such as RGS4 and RGS12, acting as GAPs (GTPase-activating proteins)[32, 86, 89, 131,340]. Furthermore, our parallel investigations compiled which regions of the Gα subunit are known to exhibit a GEF, GAP, GDI and/or GEM-like activity. Known crystal structures of the complexes Gαi-linear GEMs (KB-752 and GIV peptides[9]) underlined the significance of the SWII/α3 (switch) region within the Gα subunit.

Altogether, it can be concluded that the Gαi/s protein-directed drug development is feasible through the design and development of selective and potent compounds, such as peptides and proteins. As one approach, cell-permeable synthetic peptides (linear or macrocyclic) derived from high throughput screening procedures and subsequent chemical modification can be applied to regulate the GTPase activity of Gαi/s protein subunits. This idea inspired us to implement the strategies described in chapters 5 and 6.

2.3 Author Contribution

The co-author Anna Pepanian analyzed the literature, prepared the figures, and wrote the main text and supplementary information sections on Gαi/s-nucleotide, Gαi/s-Gβγ and Gαi/s-effector interfaces, and approaches targeting utilizing antibodies, under the supervision of Prof. Dr. Diana Imhof and with contributions of Dr. Britta Nubbemeyer and Dr. Ajay Abisheck Paul George.

3. Fluorescence Anisotropy Assay with Guanine Nucleotides Provides Access to Functional Analysis of Gαi1 Proteins

Research article

Authors: **Anna Pepanian***, Paul Sommerfeld*, Renata Kasprzyk*, Toni Kühl, Furkan Ayberk Binbay, Christoph Hauser, Reik Löser, Robert Wodtke, Marcelina Bednarczyk, Mikolaj Chrominski, Joanna Kowalska, Jacek Jemielity, Diana Imhof, and Markus Pietsch

This peer-reviewed article was published in *Analytical Chemistry*.

Citation: *Anal. Chem.* (**2022**), 94(41), 14410–14418, doi: 10.1021/acs.analchem.2c03176.

3.1 Introduction

Heterotrimeric G proteins function as binary molecular switches in order to activate various intracellular signaling cascades in response to the activation of G protein coupled receptors (GPCRs)[1]. Specific modulation of the activity and signaling of these proteins can be achieved by developing chemical tools like peptides[117,182], nucleotide analogs[16,17] or accessory proteins[172,341]. To date, great interest is focused on exploring new compounds targeting and manipulating the Gαi/s-mediated signaling pathways. The Gαi protein subunit has been examined for decades with various guanine nucleotides to elucidate the activation mechanism and Gα protein-dependent signal transduction, because the crystal structure model of Gαi was among the first structures of Gα proteins resolved[36,122]. Thereby, the presence of phosphate and magnesium ions is of paramount importance for the high affinity binding of the guanine nucleotide to the Gαi protein subunit.

In order to efficiently target and modulate the Gαi signal transduction, the production of a correctly folded and functional protein needs to be assured. Although the production of the Gαi protein has been described to be performed in various expression systems (prokaryotic[220,222,342] and eukaryotic[91,151,266,343]), many studies also reported a low protein yield[125,226] and poor solubility[225,254]. However, among all Gα protein subunits and expression systems reported so far, the bacterial production of the Gαi1 protein subunit was found to be the most time- and cost-efficient[220,225,254].

Furthermore, the direct and accurate determination of the active Gαi1 protein fraction is pending. Trypsin protection assays provide only indirect information on the protein's activity[15,225], whereas binding and kinetic studies with radioactivity-based assays are gradually substituted by more environmentally friendly and sensitive methods[207,215,344]. Such examples are fluorescence assays to determine binding affinity between the Gαi1 protein and the guanine nucleotide analog, such as BODIPY FL GTPγS or MANT-GTPγS[16,17,50,125,215,225]. Alternatively, fluorescence anisotropy (FA) is more and more utilized as a ratiometric method to investigate protein-protein interactions[344–346]. Due to the spectral properties of MANT-GTPγS restricting its applicability for FA, the second objective of this work is the development of

novel guanine nucleotide analogs binding of the Gαi1 protein subunit with high affinity (dissociation constant K_D in the low nM range).

3.2 Summary and Outlook

The first objective of this publication is the establishment of an optimized and efficient protocol for bacterial Gαi1 protein production[347]. In our studies, we used a 6xHis-tagged human Gαi1 protein, which was expressed in a *E. coli* bacterial system using existing expression protocols with various modifications. The protein expression was induced with 250 µM IPTG (isopropyl β-D-1-thiogalactopyranoside) at relatively low temperature (30 ˚C). This represented a 2.2 – 5-fold faster approach compared to the earlier reported ones[220, 222,236]. Within two purification steps, we were able to retrieve >95% pure Gαi1·GDP in 10 h and a yield of ~40 – 50 mg L^{-1} bacteria culture, exceeding reported yields[125,220,225,226]. The protein was biochemically and bioanalytically characterized with several analyses, including SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), mass spectrometry, and CD (circular dichroism). The latter method revealed a predominant α-helical character of the protein (41%) compared to the β-sheets (11%), which was in good agreement with the previous reports[134].

Second, we monitored the binding of fluorescently labeled guanine nucleotide analogs to the Gαi1 protein exploiting their spectral properties via fluorescence and FA-based assays. As probes with more favorable spectral features that can lead to high and selective binding were required, 5'/6'-carboxyfluorescein (FAM)-based guanine nucleotide analogs were synthesized in this study. As a control, the BODIPY FL GTPγS (probe 2 in the publication) was used along with nine novel FAM-probes (probes 13 – 21). The K_D values were determined upon addition of protein with the respective probe at different times of incubation. Measurements of the total fluorescence of probe 2 in complex with Gαi1 revealed a K_D value of 251 ± 78 nM (similar to the described one[17]). Probes 17 – 21 exhibited the highest binding affinity among all probes studied (~2–5 nM in the FA assays). Additional microscale thermophoresis/temperature-related intensity change (MST/TRIC) measurements were performed confirming the results. These probes are GTP-analogs with elongated linkers and showed increased binding affinity compared to their shorter or GDP-bound counterparts. The computational analyses supported this observation implying the fitting of the linkers into the nucleotide cavity. For further studies, we proceeded with probes 17 and 19 as high affinity protein binders.

Inspired by previous reports[348], FA-based assays were used to determine the active fraction of the Gαi1 protein. Therefore, we investigated two aspects: i) the concentration of Gαi1 is less than the probe concentration ensuring that the protein is fully bound to the nucleotide and ii) the opposite case, where the protein concentration exceeds the probe concentration. In this way all probes are protein-bound. Intersection of these linear regressions represents the total protein concentration assuming a 1:1 ratio. The calculations indicate a 0.69 – 0.87 of active protein fraction upon addition of probe 17 and 0.75 – 0.88 for probe 19. Most assays were performed with two different protein lots supporting the reproducibility and robustness of the approach. Finally, the GTPase-Glo assay facilitated the

quantification of the GTPase activity of Gαi1 protein to 0.95 ± 0.14 pmol GTP $pmol^{-1}$ Gαi1 with specific activities in the range of 0.016 – 0.018 min^{-1}.

Taken together, the present study provides an easy, rapid, and straight-forward strategy to obtain highly pure, soluble, and active human Gαi1 protein. Probes 17 and 19 were discovered as very high affinity binders of the protein (very low nM range). The established FA-based assays allow the investigation of Gαi modulation by nucleotide analogs in a setup comparable to the broadly used radioligand binding assays, omitting though the hazard of radioactivity. We propose the application of the newly introduced methodology also for other Gα protein subunits.

3.3 Author Contribution

*Anna Pepanian, Paul Sommerfeld, and Dr. Renata Kasprzyk contributed equally to this publication and share first-authorship.

The author Anna Pepanian analyzed the literature, established the protocols, and performed the Gαi1 protein production (expression and purification), bioanalytical characterization and overall protein handling for all applied measurements. The fluorescence and FA-based assays were performed at the laboratory of Prof. Dr. Markus Pietsch (University Hospital of Cologne, Germany). The author contributed, equally and on-site, to the performance of the experiments of the fluorescence, FA, MST, and GTPase-Glo assays and participated in scientific discussions with Paul Sommerfeld. The author provided scientific evaluation to the computational studies performed by Furkan Ayberk Binbay. The author prepared the figures and wrote the main text and supplementary information in cooperation with Paul Sommerfeld, Prof. Dr. Diana Imhof, and Prof. Dr. Markus Pietsch.

The synthesis, chemical and structural analysis of the nucleotides was performed by Dr. Renata Kasprzyk and Dr. Joanna Kowalska in the laboratory of Prof. Dr. Jacek Jemielity (University of Warsaw in Poland).

4. In-depth Characterization of Gαs Protein Activity by Probing Different Guanine Nucleotides

Research article

Authors: **Anna Pepanian**, Paul Sommerfeld, Furkan Ayberk Binbay, Dietmar Fischer, Markus Pietsch, and Diana Imhof

This manuscript is currently in review in *Protein Science*.

4.1 Introduction

The guanine nucleotide binding pocket constitutes the core region for the modulation of the GTPase cycle of heterotrimeric G proteins. The cavity is located between the α-helical (AHD) and the GTPase-domain of the Gα protein subunit. At the native, "off" state, guanosine diphosphate (GDP) is bound, keeping the heterotrimer Gαβγ complex intact and bound to the GPCR. The exchange of the nucleotide to the triphosphate counterpart (GTP) results in conformational changes in both AHD and GTPase-domains where switch regions SWI-III are mainly associated with. The result of this exchange is the activation of the G protein, dissociation from the receptor and release of the free monomeric Gα protein. A thorough investigation of this pocket and the underlying mechanism has been in the spotlight for decades providing vital experimental and structural information on the nucleotide exchange modulation. Among the four subfamilies of the Gα protein, Gαs/olf, Gαi/o, Gαq/11, and Gα12/13, Gαi and Gαs have been extensively examined since both subunits are involved in the intracellular production of the second messenger cAMP. Gαs stimulates the adenylyl cyclase (AC) production and subsequently increases the intracellular levels of cAMP, whereas Gαi functions in a reversed direction. Additionally, dysregulation of the signaling pathway of both protein subunits has been described for the progression of acute diseases. Thus, modulation of Gαi/s-mediated signaling, targeting either directly on the nucleotide binding pocket or via peptidyl ligands is a tantalizing approach.

Although both protein subunits have been expressed in bacteria cells[134,212,220,222,224,225,250], production of high amounts of the soluble and active Gαs was not as successful as of the Gαi protein[225,254]. The reduced protein solubility has been attributed to the formation of inclusion bodies, i.e. aggregated forms of the protein[225], and was described to be associated with the flexibility of the α-helical domain (AHD)[126,135]. The resulting low amounts of the expressed Gαs restricted studies for protein characterization for many years[220, 225,254]. Therefore, the Gαs activity was evaluated either upon protein modification (i.e., myristoylation[17]) or in the presence of the Gβγ[56,212,224], or β_2-adrenoreceptor (β_2-AR)[48–50,126,263]. For the determination of the Gαs activity, analyses with radioactive GTPγS isotopes[212,220,222, 224,225,250] or indirect assays (such as trypsin digestion[225]) as applied for Gαi, were conducted. Then, fluorescently-labeled GTP analogs (such as MANT-GTPγS or BODIPY FL GTPγS)[50,128,241] were utilized, providing the first hints on nucleotide binding, lacking though the determination of the actual protein activity.

In addition to the above points, the Gαs protein has also been distinguished for its heterogeneity. Already in the 1980's[212,240,242], researchers indicated the ubiquitous expression of two spliced variants of Gαs, namely Gαs(short) and Gαs(long). Both homologs differ in an area close to the linker I (between the AHD and the GTPase-domain), since Gαs(long) is longer by an additional 14mer amino acid sequence. Initial studies designated a faster rate of the dissociation of GDP from the Gαs(long) version[186,212,224]. However, most analyses and experiments were primarily performed for the short variant[17,22,123,126,128,162,225,257,258]. On the molecular and structural level, the X-ray structural analysis of Gαs(short)·GDP (PDB:6EG8[48]) and Gαs(short)·GTPγS (PDB:1AZT[44]) was performed using the short isoform, whereas for the Gαs(long) protein, only an AlphaFold structure (Uniprot ID: P63092-1) is available.

Notably, Novotny *et al*.[186] summarized and denoted the controversies regarding the functional assessment of the two isoforms, with inconsistent effects on the AC activity being reported over the years[196,243,349]. So far, however, no further experimental, or structural studies have provided further detailed information to enable the exploration of their functional differences. For these purposes, an approach that can substantially investigate, characterize, and provide an accurate insight into the activity of the G protein required.

4.2 Summary and Outlook

Driven by previous reports on the low yield of soluble human Gαs obtained from recombinant protein expression, the first intention of this manuscript is the development of an approach producing high yields of properly folded recombinant, human Gαs(long) and Gαs(short) proteins[350]. Herein, both isoforms were unmodified (no myristoylation or palmitoylation), carrying only a 6xHis-tag at the N-terminus. Proteins were expressed in *E. coli* cells and purified in two steps (affinity and size exclusion chromatography) using existing, yet slightly modified protocols[126,128,129,220]. Different concentration of IPTG were tested with 30 µM isopropyl β-D-1-thiogalactopyranoside (IPTG, 30 ˚C for 9 h) revealing the highest efficiency among all. The long isoform was more prone to aggregation and thus, a refolding protocol was generated and applied[134,225,351]. The established protocols resulted in 2.5 - 2.9 mg L^{-1} native Gαs(long)·GDP, 5.0 – 6.1 mg L^{-1} refolded Gαs(long)·GDP, and 4.0 – 4.8 mg L^{-1} native Gαs(short)·GDP. Although these values are superior to previous approaches, the same methodology was 10-fold more efficient for the Gαi1 protein[220,347], verifying the challenging production of the Gαs protein. The protein was biochemically and bioanalytically characterized with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), mass spectrometry, Edman sequencing and CD (circular dichroism). As aforementioned (chapter 4.1), there is no structural information available for the long isoform, limiting the comparison of the experimental data with existing literature. Therefore, basic computational studies were applied to compare the structures of the Gαs isoforms[48] and the Gαi[122] protein. Only for the Gαs(short)·GDP the CD data indicated a predominant α-helical character (31%) compared to the β-sheet content (18%) which is in agreement with previous reports[134]. Regarding the long isoform, a lower α-helicity (21%) was observed, and it seems that the protein adopts a more random

conformation with a higher β-sheet character (25%). This observation was also supported by computational studies where conformational changes within AHD were observed. In contrast, the refolded Gαs(long)·GDP showed a complete loss of the α-helical conformation.

Literature screening indicates a generally higher binding affinity of the Gαi protein for nucleotide analogs compared to Gαs[30, 212,352], with only one exception, i.e. the report of McEwen *et al*.[17], that stated a higher binding of BODIPY FL GTPγS to myristoylated Gαs(short) compared to Gαi (70 nM vs. 150 nM). Since this value and observation was not reproduced since, the second objective of this manuscript was the investigation of the binding between Gαs(long)/(short) with different nucleotide analogs. The same setup of fluorescence and fluorescence anisotropy (FA) assays was utilized, as was previously described for Gαi[347]. Initially, the BODIPY FL GTPγS probe was tested indicating a fast ligand binding (~120 s for the Gαs(long) and ~240 s for the Gαs(short) isoform). Time-dependent changes of fluorescence and FA intensities suggest the formation of a less stable complex due to low intensities reported after time, presumably resulting from dissociation of the fluorescent species from the protein. Apart from the BODIPY FL GTPγS, MANT-GTPγS and two nucleotide probes with high binding affinity for Gαi1 (i.e., probes 17 and 19) were tested. Among all probes, BODIPY FL GTPγS was the most efficient with binding affinities of 1.6 – 1.9 µM for Gαs(short) and 5 – 6 µM for Gαs(long) based on fluorescence and FA intensities, respectively. Additional studies point to the prevention of BODIPY FL GTPγS binding to Gαs(short) in the presence of increasing concentrations of GTPγS, supporting the stronger binding of GTPγS as previously shown[49,50].

Molecular dynamics (MD) simulations studying the interactions between both Gαs isoforms with the probes GTPγS, BODIPY FL GTPγS, MANT-GTPγS, 17, and 19, were also performed and reported for the first time. The results verified the experimental data, in which GTPγS and BODIPY FL GTPγS are the most efficient binders for both Gαs isoforms. Finally, the studies highlight the flexibility, not only of the protein's AHD, but also of the fluorophore moiety (BODIPY FL) of the BODIPY FL GTPγS probe, which could explain the decrease in binding affinity between protein and nucleotide ligand over the time.

Finally, the GTPase activity of Gαs(long) and Gαs(short) was investigated utilizing the GTPase-Glo assay as previously established[347,353]. According to the linear interdependency of GTP and protein concentration, the specific activities of Gαs(long) and Gαs(short) were determined to be 1.7 min^{-1} and 0.13 min^{-1}, respectively. These values are somewhat in the range described before[22,212,220], indicating a faster GTPase hydrolysis and multiple GTP turnover for the long isoform.

Altogether, this manuscript provides an efficient protocol for the production of higher amounts (than previously reported) of soluble, unmodified, recombinant human Gαs(long) and (short) protein subunits expressed in bacteria cells. The conformational and structural investigation of both isoforms with experimental and computational studies is reported for the first time and verifies previous statements of AHD flexibility. The functional differences between the long and short homologs based on their binding affinities to several nucleotide probes, suggest the GTPγS and BODIPY FL GTPγS as efficient probes

for the Gαs protein, with a higher affinity being confirmed for the short isoform. In parallel, probes 17 and 19 were found to be selective high affinity binders for Gαi1^{347}, since their affinity to Gαs was significantly lower. For the future, the above protein expression approach followed by the functional characterization setup, could be further applied to investigate other Gα protein subunits.

4.3 Author Contribution

The author Anna Pepanian analyzed the literature, established the protocols, and performed the Gαs (short and long isoforms, both soluble and refolded) protein production, bioanalytical characterization, and general protein handling for all applied measurements. The author contributed to the experiments concerning fluorescence, fluorescence anisotropy and GTPase-Glo assays and participated in scientific discussions with Paul Sommerfeld for data analyses. The author assisted in the evaluation of the computational studies performed by Furkan Ayberk Binbay. The author prepared the figures and wrote the main text and supplementary information in cooperation with Paul Sommerfeld, Furkan Ayberk Binbay, Prof. Dr. Markus Pietsch, and Prof. Dr. Diana Imhof.

5. Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators

Research article

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This peer-reviewed article was published in *ACS Chemical Biology*.

Citation: *ACS Chem. Biol.* (**2022**), 17(2), 463–473, doi: 10.1021/acschembio.1c00929.

5.1 Introduction

Numerous approaches for directly targeting GPCRs have been successfully conducted and led to a variety of drugs, but also to valuable pharmacological tools. However, this status has not been achieved for G proteins yet. Therefore, the development of suitable chemical tools for modulating the Gαi/s-mediated signaling cascades in a GPCR-dependent as well as independent manner is a focal point of research today. Two cyclic depsipeptides, namely FR900359 (FR) and YM-254890 (YM), were discovered to have an inhibitory function on the Gαq protein subunit, whereas linear peptides, such as KB-752[12] and R6A[326] were milestones possessing a GEF (guanine nucleotide exchange factor)-like function for the Gαi1 protein. The KB-752 peptide shares sequence similarity with the GIV-GEM motif, which resulted not only in the GEM (guanine exchange modulator)-like bifunctional activity on Gαi/s proteins, but also confirmed their binding site on the protein, i.e., the SWII/α3 region. This hydrophobic cleft has been shown to be the site for high affinity binding of peptides and is also involved in the interaction with effector molecules, such as AC[44,51,123]. Finally, this area impedes interactions between the Gα monomer with the Gβγ dimer, impeding the heterotrimer re-association[85,89,203].

Combinatorial peptide library screening techniques are innovative and useful tools used as a starting point for identifying potent Gα protein modulators. They provide variable candidates due to their high diversity. Subsequent optimization of the identified hit compounds is usually facilitated by chemical modification, such as (macro)cyclization or addition of a CPP (cell-penetrating peptide) motif, to enhance the cell permeability and metabolic stability of the ligand[277,298,330,332,354,355].

5.2 Summary and Outlook

In this peer-reviewed article, we performed the screening of a one-bead-one-compound (OBOC) combinatorial peptide library against the Gαi1·GDP subunit and discovered novel linear peptides. The positive hits were characterized by (partial Edman degradation) in combination with MALD mass spectrometry (PED-MALDI MS) and a consensus sequence was derived. Among all peptides, the linear nonapeptide GPM-1 exhibited sequence similarity to the aforementioned KB-752, GIV-GEM, and R6A peptides. Binding studies (MST and SPR) between the peptide and Gαi1/s·GDP showed a high affinity

of the ligand to the proteins (~0.14 μM for Gαi1·GDP and ~0.63 μM for Gαs·GDP). Before we proceeded with activity testing of the peptides in cellular assays, further chemical optimization of the lead compound GPM-1 was required to facilitate cell penetration. Therefore, the derivatives GPM-1b (cyclic analog of GPM-1), GPM-1c (GPM-1 conjugated N-terminally with the cell-penetrating peptide (CPP) motif F-2Nal-RRRR (F: L-phenylalanine; 2Nal: L-2-naphthylalanine) and GPM-1d (cyclic GPM-1c) were synthesized and their binding to the respective proteins was verified.

Biological studies with two approaches, a membrane-based and a whole-cell assay, indicated the GEF-like activity of GPM-1c and GPM-1d to Gαi1 and a GDI-like activity to Gαs. Thus, GPM-1 and its derivatives could serve as the first linear and cyclic peptidyl modulators derived from an OBOC combinatorial library, possessing a GEM-like activity for Gαi1/s proteins. Molecular dynamics simulations supported the GEM-like function, since GPM-1 and its analogs showed a favorable binding pose in close proximity to the common SWII/α3 region.

Aa a result of this study, the screening of an OBOC combinatorial library was successful concerning the development of novel peptide scaffolds with biological prominence for Gαi1/s-mediated signaling modulation. Thus, we suggest that OBOC and presumably OBTC (one-bead-two compound) libraries (see chapter 1.4) can be utilized for the discovery of G protein modulators. Finally, the lead compound GPM-1 could be further optimized and tested for medicinal relevance (pharmacological assays).

5.3 Author Contribution

The author Anna Pepanian performed the Gαi1/ Gαs protein production (expression and purification) and bioanalytical characterization. The author prepared the figures and wrote the main text and supplementary information with regard to the protein preparation and handling under the supervision of Prof. Dr. Diana Imhof.

The OBOC combinatorial library screening, the respective peptide synthesis and characterization as well as the binding studies (MST and SPR) were performed by Dr. Britta Nubbemeyer, under the supervision of Prof. Dr. Diana Imhof. The library screening was in collaboration with Prof. Dr. Dehua Pei (the Ohio State University, Columbus, USA). The biological assays were performed by Dr. Eva Marie Pfeil, Dr. Suvi Katariina Annala, Dr. Maximilian Muehlhaupt, and Prof. Dr. Hermann Ammer. The computational studies were performed by Dr. Ajay Abisheck Paul George, and Rahma Maghraby.

6. Bicyclic Peptide Library Screening for the Identification of Gαi Protein Modulators

Research article

Authors: **Anna Pepanian**, Furkan Ayberk Binbay, Suchismita Roy, Britta Nubbemeyer, Amritendu Koley, Curran A. Rhodes, Hermann Ammer, Dehua Pei, Pradipta Ghosh, and Diana Imhof

This manuscript has been accepted for publication in the *Journal of Medicinal Chemistry*.

6.1 Introduction

Synthetic G protein modulators that can function as chemical tools targeting selectively the Gα subunit in a receptor-independent manner are desired in order to obtain a better understanding of the G protein-mediated signaling[20,174,182,326]. In our first series of peptides[21] (as described in chapter 5.1), we developed a lead compound, i.e. the linear nonapeptide GPM-1 (G protein modulator), derived from an OBOC combinatorial peptide library screening against the Gαi1 protein subunit. In addition, chemical modifications, such as the introduction of a cell-penetrating peptide (CPP) motif or head-to-tail cyclization of GPM-1, resulted in the derivatives GPM-1c and GPM-1d which possess even greater binding affinity and biological activities, exhibiting a GEM-like activity upon stably binding to the earlier introduced SWII/α3 region (chapters 1.1.2, 1.2.1, 1.3, and 5).

Previous studies utilizing an one-bead-two compound (OBTC) combinatorial peptide library were successful in identifying inhibitors for the small molecule K-Ras[18,24]. This peptide library facilitates the development of macrocyclic peptidyl compounds, which, compared to the linear counterparts (chapter 5.1), provide enhanced structural rigidity. In addition, the introduction of unnatural or D-amino acids is favourable for augmented cell permeability and stability towards proteolytic degradation[293,295,298,319,331,356,357]. Macrocyclic peptides, such as the cycGiBP[332], cycPRP-1[333], cycPRP-3[333], and Gα SUPR[296], have been identified as binders of Gαi1·GDP (inactive state), lacking though further structural information. Also, the lack of adequate cellular studies limits their further investigation and classification into the known modulator categories (GEFs, GDIs, GAPs, and GEMs, see chapter 1.1.1 and 1.4.2). Although there were already some interesting compounds available (e.g., KB-752, GIV, and our GPM-1), no other compound possessing e.g., a GAP-like activity, as is known for RGS proteins (see chapter 2.2), was described for Gαi/s so far.

An additional and important point here is the deficiency of compounds targeting both states of the Gαi1 protein subunit individually. With only one exception, i.e. peptides that are Gαs state specific[22], there is no report on peptidic ligands addressing the active Gαi1 state available thus far. For this reason, strategies developing specific ligands for the Gαi1·active state by employing GMPPNP, the non-hydrolyzable nucleotide analog of GTP, are highly desired.

6.2 Summary and Outlook

This manuscript outlines the application of an established OBTC (one-bead-two-compound) combinatorial peptide library for screening against both states of the Gαi1 protein subunit (by using Gαi1·GDP and Gαi1·GMPPNP). Initially, out of $5.4 \cdot 10^5$ beads, thirty-five beads for Gαi1·GDP and thirty-six beads of Gαi1·GMPPNP were isolated and further analyzed to identify their sequences with the aforementioned method PED-MALDI MS (chapter 5). This reduced the number of selected beads to twelve and fifteen hits for the inactive and active state, respectively. A sequence alignment and analysis of the amino acid frequency resulted in four peptide candidates (1 – 4, numbering according to the manuscript) for the inactive Gαi1 and four for the active state (5 – 8).

The verified peptide candidates were resynthesized via solid-phase peptide synthesis (SPPS), and their biochemical and bioanalytical characterization with the respective protein state was followed. Surface plasmon resonance (SPR) analyses suggested peptides 2 and 3 (GPM-2 called thereafter) as efficient Gαi1·GDP binders and peptide 8 (GPM-3) as a Gαi1·GMPPNP binder with affinity in the low µM range (e.g., 0.71 µM) which is adequate for such constrained chemical structures. Also, this came in agreement with membrane-based assays on NG108-15 membrane preparations, in which the intracellular cAMP levels were obtained as the final readout. The peptide GPM-2 led to ~40% cAMP decrease and GPM-3 to a 30% increase, implying the contradictory function between the two compounds. Moving further from the membrane to the interior of the cells, we conducted GPCR-independent BRET (Bioluminescence Resonance Energy Transfer) assays with the promising peptide candidates 2, GPM-2, and GPM-3. In the first set-up, cAMP levels upon forskolin (Fsk) stimulation were measured. GPM-3 showed a ~10% elevated cAMP, and GPM-2 a cAMP reduction (with lower potencies), similar to the membrane assays. The modest activity of some of the peptides can be an outcome of low cell permeability and therefore, further optimization, such as CPP conjugation could be considered in the future. In parallel, MTT and plasma stability assays indicated the cell viability and the proteolytic stability of the lead compounds, e.g., GPM-3. Despite the absence of a CPP-conjugation, GPM-3 was able to penetrate the cell membranes and be metabolically intact. Concluding, the GPM-3 compound targets the active state of the Gαi1 protein and leads to elevated cAMP levels. Therefore, we hypothesized that it possibly attains a GAP (GTPase-activating protein)-like activity by inactivating the Gαi1-mediated signaling. End-point cell assays enabled the quantification of the secreted cAMP (IC_{50} value of 6.92 µM). On the contrary, GPM-2 was suggested to exhibit a GEF (guanine nucleotide exchange factor)-like activity due to the decreased cAMP levels detected. The peptides were inactive for Gαs signaling, highlighting the selectivity of the compounds for their specific protein.

In order to dig deeper into our GAP theory for GPM-3, two additional BRET set-ups were applied investigating the heterotrimer (Gαβγ) reassociation. The first one aimed at the GRK3 (C-terminus of G protein-coupled receptor kinase-3) detection, an indicator of released free Gβγ. The second BRET system detected free monomeric Gα protein, which is again an indicator of heterotrimer dissociation. Both measurements on GPM-3 displayed no elevation for neither free Gα monomer nor GRK3 (or

subsequently Gβγ dimer), enhancing our assumption that the peptide may evoke the Gαβγ re-association.

The experimental observations were underpinned with intense computational analyses. Since we did not have results on binding poses of other cyclic peptides to the G proteins, we investigated the known areas for Gαi modulation (such as SWII/α3). Novel binding poses for both GPM-2 (on Gαi1·GDP, homology model from reference[21]) and GPM-3 (on Gαi1·GTPγS, PDB ID: 1GIA[122]) were identified. Bond pairing between Arg7-Glu238, Phe8-Glu238, Dap10-Ala235 and Dap10-Glu236, and Lys11-Asp237, were observed at frequencies >80%. The residues Ala235, Glu236, and Asp237 are commonly involved in GTP hydrolysis. Moreover, we observed perturbation of the G-R-E triad (see chapter 1.1.2), an indicator for potential heterotrimer re-association.

All things considered, we obtained two different novel lead compounds from an OBTC combinatorial peptide library screening, namely GPM-2 and GPM-3. Both compounds are class and state-selective ligands binding to the respective protein state with competent affinity and novel sites (topological investigation). Experimental and computational studies suggest GPM-3 as the first GAP-like modulator of Gαi1·GMPPNP (active state), penetrating the cell membrane and increasing cAMP levels at very low concentrations (2.5 µM). Basic pharmacological pre-testings excluded cell toxicity or cleavage by plasma proteases. GPM-2 exhibited a potential GEF-like activity, however further studies aiming at higher cell permeability would be beneficial.

6.3 Author Contribution

The author Anna Pepanian established the protocols and performed the Gαi/s protein production (expression and purification), bioanalytical characterization and general protein handling for all applied measurements. The peptide synthesis and analytical characterization was performed by Dr. Britta Nubbemeyer with support from the author. The author contributed equally to the experimental conduction and evaluation of all cellular assays (BRET, FRET, MTT and ERK/Akt-signaling assays) together with Dr. Suchismita Roy during a research stay (on site) at the laboratory of Prof. Dr. Pradipta Ghosh (University of California San Diego). The author assisted in the evaluation of the membrane-based assays performed by Prof. Dr. Hermann Ammer, and of the computational studies performed by Furkan Ayberk Binbay. The author prepared and performed the SPR and plasma stability assays, analyzed the literature, prepared the figures, and wrote the main text and supplementary information under the supervision of Prof. Dr. Diana Imhof.

7. Summary

The G protein coupled receptors have been broadly investigated as pharmacological targets in several pathophysiological conditions, with more than 30% of the FDA approved drugs addressing these receptors[3,14]. GPCRs are communicating with the cells through the membrane bound heterotrimeric Gαβγ proteins. Since the G proteins are located in the interior of the cells, they are not easily accessible by external molecules for direct binding and therefore a GPCR-dependent, i.e., canonical, G protein activation mechanism is commonly taken place. The Gα protein subunit is further classified into the Gαi/o, Gαs/olf, Gαq/11 and Gα12/3 subfamilies[1]. In the inactive, native state, the heterotrimeric G protein carries the GDP nucleotide on the Gα subunit (between the AHD and the GTPase domain). Upon agonist binding to the GPCR, the receptor associates with the G protein and induces internal conformational changes (mainly in the SWI-III regions[34–36]). The heterotrimer loses its affinity for GDP and intracellularly abundant GTP is binding. The transition state, where no nucleotide is bound, leads to heterotrimer dissociation into two distinct units, the monomeric Gα and the Gβγ dimer[32]. Afterwards, downstream effectors are subsequently affected. For instance, the Gαs stimulates, whereas the Gαi1 subunit inhibits the intracellular AC and subsequently cAMP production[57–59]. The latter one is an important second messenger in several signaling cascades. Therefore, any implication of the physiological function of these proteins could lead to severe diseases, such as asthma[7,8], heart failure[104,105] or even cancer[97,109].

In the second chapter of this thesis, the review[5] summarizing the knowledge of more than 40 years concerning Gα protein analysis and function is described, highlighting the most important interfaces of the Gαi/s proteins with their up- and downstream effector molecules to support the future development of suitable and potent pharmacological tools. Indeed, G proteins are versatile since they share interfaces for the contact with the GPCR, the downstream effectors, the Gβγ dimer for complex association and dissociation, the guanine nucleotide pocket, and accessory proteins. As described for the GTPase cycle, the core function of the G proteins derives from the nucleotide binding. The nucleotide pocket though is located ~30 Å away from the receptor-protein interface requiring only allosteric interactions for changes of this cavity[129,166]. The protein-effector (i.e., AC) interface was also studied throughout the years revealing molecules that can assist experiments aiming the intracellular cAMP observations. Among these molecules are Fsk[192,193], Iso[197–199], and DADLE[358], which facilitate intracellular cAMP measurements. The function of these molecules was exploited for the two studies described in chapters 5[21] and 6[140]. Moving on to the protein-nucleotide interface, the modulation can be indirectly addressed. Although nucleotide analogs cannot be proposed as drugs, they can be used for evaluating the activity of the protein even in the context of receptor association. Nucleotide probes, such as BODIPY FL- or MANT-GTPγS, have been previously tested and used for evaluating the AC stimulation after association with the β_2-AR[16,17,49,50,128,241]. Yet, methods for accurate determination and quantification of human Gαi/s proteins are still to be established.

Because of this, adequate protein modulation cannot be studied without confirmed functional protein material. Since the present dissertation aimed at making progress concerning Gαi/s protein modulation, the initial studies on the Gαi1 (chapter 3[347]), and Gαs(long) and Gαs(short) protein isoforms (chapter 4) were the starting with the implementation of suitable production protocols. Molecular biological studies on the Gαi/s subfamilies go back to the 1980's[13,30,242]. Since then, a plethora of researchers expressed Gαi/s in different expression systems (eukaryotic and prokaryotic). Although *E. coli* bacteria cells were not fruitful for Gαq and Gα12 subfamilies[14,124,227,228], they were more promising for Gαi/s, however, are still to be optimized.

An analysis of the existing expression and purification protocols from the literature led us to established a modified approach with e.g. lower induction temperature and higher IPTG concentration, which resulted in a 4-fold faster and 9-fold more efficient (in terms of yield) approach for Gαi. Conventional biochemical and bioanalytical characterization was applied to verify the purity and fold of the protein sample, revealing ca. 41% α-helical secondary structure and thus confirming earlier findings. Novel fluorescently and non-radioactive labeled nucleotide analogs, i.e. compounds 17 and 19[347], were developed and found to bind to the Gαi1 protein with very high affinities (2-5 μM). Fluorescence and fluorescence-anisotropy (FA) assays were performed to provide a direct and quantitative protein evaluation[359]. The fluorescence-based assays were established at the laboratory of Prof. Dr. Markus Pietsch (University Hospital of Cologne, Cologne, Germany). The FA setup was further utilized to determine the active protein fraction[348]. At this point we would like to emphasize that our work provided not only high amounts of soluble protein, but also highly active protein fractions (~70 – 90%) upon binding of the probes 17 and 19. These probes are significantly more effective than the broadly used BODIPY FL GTPγS. The GTPase-Glo assay indicated a specific activity of 0.016 – 0.018 min^{-1} and 95% of our protein was able to bind GTP.

Next, the above methodology was applied to the other protein subunit under study, the Gαs (chapter 4). The Gαi/s proteins share a sequence similarity of 40%[29] (Figure 1) and although they target a common effector molecule, i.e. AC, their expression profile and subsequent analysis is rather different. Numerous studies reported low yields and solubility issues as well as, in some cases, misfolding[225,254]. There are two homologs of the Gαs subunits derived from evolutionary mRNA splicing variants, the long and the short isoform[242], the functional difference of which is rather complicating to unravel[186]. Our studies provide an optimized protocol for the production of higher amounts compared to previous reports[212,220, 222,225,250], of soluble, unmodified, recombinant human Gαs(long) and (short) protein, expressed in a bacterial system. The purity, sequence and secondary structure were verified with biochemical and bioanalytical methods. Circular dichroism (CD) and computational data were, for the first time, published for the long isoform indicating a rather conformationally flexible AHD. Fluorescence and FA-based assays indicated the preference of Gαs(short) and (long) binding to the BODIPY FL GTPγS probe (K_D values of 1.6 – 1.9 μM for Gαs(short) and 5 – 6 μM for Gαs(long)) compared to MANT-GTPγS, 17 and 19 probes. The binding affinity is reduced upon increasing amounts of GTPγS, which functions in these cases as a good competitor. Computational studies verified the experimental data highlighting not only

the flexibility of the AHD of the Gαs protein, but also the flexibility of the fluorophore dyes (BODIPY FL and MANT). As for the Gαi1 protein, GTPase-Glo assays were performed revealing specific activities of Gαs(long) and Gαs(short) of 1.7 min^{-1} and 0.13 min^{-1}, respectively. This study not only provided vital information on the conformation of both Gαs isoforms but also about their functional behavior regarding guanine nucleotide exchange.

Considering the G protein interfaces, the accessory protein-G protein interface includes the most promising hints for Gαi/s protein modulation[210]. As already described by their names, accessory proteins are proteins supplementing distinct pathways or mechanisms. Guanine nucleotide exchange factors (GEFs) enhance the GDP-GTP exchange, GDP dissociation inhibitors (GDIs) stabilize the protein in the inactive state via inhibition of GDP dissociation, and GTPase activating proteins (GAPs) induce GTP hydrolysis, heterotrimer reassociation and subsequent signal termination. A new category of modulators described in the last decade[9,80,360] are guanine exchange modulators (GEMs). The characteristic feature of GEMs is their bifunctional activity of acting as a GEF for Gαi and GDI for Gαs. Remarkably, this is only described for two compounds so far, i.e. the GIV/Girdin motif and the peptide KB-752, both sharing a ΦTΦX[D/E]FΦ sequence motif (Φ: hydrophobic residue, X: any random residue)[21,80,170,329]. Both compounds bound to the hydrophobic cleft of SWII/α3, a catalytic area involved in the interactions with AC[44,51,123] and natural accessory proteins (Ric-8[136,145,146,172] and RGS14 GoLoco motif[200,201]), but typically excluded for Gβγ interactions[9,20,22,145,155].

This GEM-like activity of the nonapeptide KB-752, which was derived from a phage-display library, inspired us to implement two studies that are described in chapters 5 and 6. Although two cyclic depsipeptides YM-254890[321] and FR900359[182,323] have been identified as selective Gαq inhibitors, such potent modulators are not yet described for Gαi and Gαs proteins. Combinatorial peptide library screening is a typical strategy for the identification of peptide scaffolds that bind to protein surfaces. Highly diverse candidates can be derived either based on DNA-encoding or synthetic libraries[301,302]. The mRNA- or phage display strategies mentioned above allow for the detection of primarily linear peptides, however, are limited to natural amino acids[310]. This is an important aspect to take into consideration for drug development where aspects such as proteolytic stability come into play. It is not only sufficient for a specific ligand to bind to the G protein of interest, but also to penetrate the cell membrane and survive proteolytic attack. To suppress these threats, the lead compounds can be further modified either by cyclization or by introduction of non-proteinogenic amino acids, and conjugation to or insertion of CPP (cell-penetrating peptide) motifs[283,286–290]. Considering these aspects, a one-bead-one compound (OBOC) library (chapter 5[21]) as well as a one-bead-two compound (OBTC) library (chapter 6[140]) were screened in consecutive studies. The first library was screened against Gαi1·GDP and resulted in the linear nonapeptide GPM-1 (RWLRYLRYP), which binds to Gαi1·GDP with an affinity of K_D 0.14 μM and to Gαs·GDP with 0.63 μM. Membrane-based assays and whole-cells assays (with HEK-293 cells) revealed a GEM-like activity (GEF for Gαi1 and GDI for Gαs). Chemical modifications of GPM-1 (GPM-1b: cyclic GPM-1 derivative, GPM-1c: GPM-1 with a CPP conjugated and GPM-1d: cyclic GPM-1c derivative) improved binding and cellular activity, as expected. Computational analyses revealed that

GPM-1 is binding to an area close to the SWII/α3 cleft enhancing our assumption that we developed the first GEM compound derived from an OBOC library.

Last but not least, the fifth work described in this dissertation is the second series of peptidyl modulators. In this approach, an OBTC combinatorial library (established in the laboratory of Prof. Dr. Dehua Pei, OSU, Columbus, USA) was employed, which was earlier successfully applied and revealed potent inhibitors for the K-Ras protein[18,24]. Additionally, we screened the library not only for the inactive Gαi1·GDP state but also for the active Gαi1·GMPPNP (non-hydrolyzable analog). Macrocyclic peptides, such as the cycGiBP[332], cycPRP-1[333], cycPRP-3[333], and Gα SUPR[296] peptide are reported only as Gαi1·GDP binders, lacking additional structural information. The introduced OBTC library can screen hits rich in aromatic and positively charged residues, and also unnatural amino acids to indirectly provide increased cell permeability and metabolic stability[294,312,322,339,340]. From the screening approach, four peptides (1–4) were identified as binders for Gαi1·GDP and four (5–8) for Gαi1·GMMPNP. These peptides were resynthesized and chemically characterized for purity and quality. Binding studies (SPR) revealed an increased affinity for peptides 2 and 4 (GPM-2) for Gαi1·GDP and GPM-3 for the active Gαi1 protein. Validation of the hits was also performed with membrane-based assays, in which GPM-2 and GPM-3 decreased and increased the cAMP production (by 30–40%), respectively. These ELISA-based assays performed are established in the laboratory of Prof. Dr. Hermann Ammer (LMU, Munich, Germany). Accordingly, we hypothesized that GPM-2 might attain a GEF-like activity, while GPM-3 acts as the first reported peptidyl GAP modulator. Cellular assays in HeLa cells were conducted via three different BRET assays (established in the laboratory of Prof. Dr. Pradipta Ghosh, UCSD, San Diego, USA). The first setup targeted the intracellular cAMP production after peptide incubation. The results of GPM-2 and GPM-3 were consistent with the membrane-based assays, with GPM-2 decreasing the cAMP levels. Assuming a lower cell penetration though, future optimizations should be considered to increase the cellular uptake. In order to verify the assumption of GPM-3 acting as a GAP, BRET signaling of the released Gβγ subunit and the free monomeric Gα protein was analyzed. Based on the results, no free moiety was detected supporting the idea of the occurrence of heterotrimer reassociation. Extended computational studies were also conducted for the peptides under study to aid visualization of the complex formation and support mechanistic explanations of the peptides' activities. To the best of our knowledge, this is the first time that bicyclic peptides targeting both states, i.e., Gαi1 GDP and GMPPNP states, were analyzed in this way. Binding poses of GPM-2 and GPM-3 on the respective protein template indicate new interaction sites compared to the previously described SWII/α3 region. GPM-3 formed stable (>80% frequency) bonds with catalytical residues involved in the GTP hydrolysis (e.g., Glu238, Glu238, Ala235, Glu236, and Asp237[36,122,144]). Perturbation of the G-R-E triad[144] would impede the Gβγ dissociation and confirming the GAP-like activity of GPM-3.

Taken together the aforementioned findings, the present dissertation encloses novel advances towards the receptor independent Gαi/s protein modulation. A thorough review description of the five different Gαi/s protein interfaces can be utilized to assess druggable sites of the protein and establish strategies for modulator development. A protocol of high yielded and soluble Gαi1 in combination with the

methodology to functionally characterize the protein with non-radioactive and highly selective FAM-based nucleotide analogs is described. For many years, the investigation of the two Gαs isoforms was paused and less recognized in the community. The protocols developed in the context of the present dissertation could overcome the low yields reported for the isoforms and enable more detailed testing of their biochemical function. The short variant exhibited higher affinity, but slower binding for the BODIPY FL GTPγS analog compared to the long isoform. The high flexibility of the α-helical domain of the long isoform and the consequences thereof should be studied more intensively in the future. Regarding the modulator development, two approaches for identifying novel linear and bicyclic peptides with promising GEM, GEF or GAP-like activity are presented. Further chemical optimization and pharmacological investigation will shed light on the capability of these modulators and derived analogs in biomedical and therapeutic applications.

8. References

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Acknowledgment

This endeavor would not have been possible without the valuable contribution of my advisor and most importantly my mentor *Prof. Dr. Diana Imhof*. Words cannot express my gratitude to her commitment, everlasting patience and faith in me and the project. Through her exceptional leading management, projects that were initially impossible turned out to become a successful story to tell. Being a part of this group, inspired me and gave me the confidence that women in science are more than role models.

I also could not have undertaken this journey without my defense committee, *Priv. Doz. Dr. Arijit Biswas* (Institute for Experimental Haematology and Transfusion Medicine, University Hospital Bonn) for honoring me as my second referee.

A special gratitude to *Prof. Dr. Karl Wagner* and *Prof. Dr. Matthias Geyer* for being members of my thesis committee. Additionally, this thesis would not have been feasible without the generous support from the Deutsche Forschungsgemeinschaft (DFG) for financing my research.

Working on this project did not only help me as a scientist but provided me with many experiences and lessons. Collaborations allowed me to gain more theoretical and practical skills. A distinguishing experience is the pleasant, despite the long-night measurements, collaboration with the PhD student *Paul Sommerfeld* and *Prof. Dr. Markus Pietsch*. Moreover, special thanks for the knowledge exchange to *Dr. Suchismita Roy* and of course *Prof. Dr. Pradipta Ghosh* for this life-changing opportunity to work overseas in a completely new field to me. I will always keep our enlightening discussions in mind.

I am also grateful to my colleagues and cohort members, for their scientific contribution, long-lasting feedback discussions, and moral support. A special thanks to *Toni* and *Marie* for devoting their time to "draw" and solve project-mysteries. I express my appreciation also to *F. Ayberk Binbay* for supporting me with his computational studies. To all my colleagues (*Yomnah*, *Sonali*, *Dhruv*, *Karina, Simona,* and *Sabrina*), a big thanks for spending quality time in our hiking days and retreat trips and hopefully our engagement was not "disturbing". Thanks, should also go to all students (especially to *Sabriye*) that I was honored to supervise and enabled me not only to complete experiments with their support, but also providing me the chance to transfer my knowledge to them and promote scientific research.

Lastly, I would like to express from the bottom of my heart appreciation and respect to my family, especially my parents, *Miranda* and *Gkarnik* (*Aris*), and my beloved sister, *Mary*, who were always there to hear about the ups and downs of my studies. Their belief in me has kept my spirits and motivation high during this process. This in combination with the emotional support of my friends, the family that I chose (*Liana*, *Katerina*, and *Christina*), was more than I would wish for. Thanks for providing me with happy and entertaining moments of relaxation and traveling.

List of abbreviations

aa	amino acid
AC	adenylyl cyclase
AGS	activators of G protein signaling
AHD	α-helical domain
AT1	angiotensin 1
ATP	Adenosine triphosphate
β_2-AR	β_2-adrenergic receptor
BCIP	bromochloroindolyl phosphate
BODIPY FL GTPγS	guanosine 5'-O-(γ-thiotriphosphate) N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacine-3-yl) methyl) thioester
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CD	circular dichroism
CPP	cell-penetrating peptide
Cryo-EM	cryogenic electron microscopy
CTX	*cholera* toxin
DAG	diacylglycerol
E. coli	*Escherichia coli*
et. al.	*et alii*
FA	fluorescence anisotropy
FAM	5'/6'-carboxyfluorescein
FR	FR900359 depsipeptide
Fsk	forskolin
GAP	GTPase-activating protein
GBA	Gα-binding and -activating protein
GDI	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GEM	guanine exchange modulators
GIRK	G protein-regulated inwardly-rectifying potassium
GIV	G-interacting vesicle-associated protein

GMPPNP	guanyl-5'-(β,γ-imino)triphosphate
G protein	guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
GRK3	G protein-coupled receptor kinase-3
GTP	guanosine triphosphate
GTPγS	guanosine-5'-O-(3-thio) triphosphate
6xHis-tag	hexahistidine tag
IP_3	inositol-1,4,5-triphosphate
iPPSD	inactivating PTH/PTHrP signaling disorder
IPTG	isopropyl β-D-1-thiogalactopyranoside
Iso	isoproterenol
K_D	dissociation constant
MANT-GTPγS	2'/3'-O-(N-Methyl-anthraniloyl)-guanosine-5'-(γ-thio)-triphosphate
MS	mass spectrometry
MST-TRIC	microscale thermophoresis/temperature-related intensity change
NMR	nuclear magnetic resonance
OBOC	one-bead-one compound
OBTC	one-bead-two compound
PDE	phosphodiesterase
PED	partial Edman degradation
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PPI	protein-protein interaction
PTM	post-translational modification
PTX	*pertussis* toxin
RaPID	random nonstandard peptide integrated discovery
ROCK	Rho-associated protein kinase
RGS	regulator of G protein signaling
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPPS	solid-phase peptide synthesis

SPR	surface plasmon resonance
SW	switch
YM	YM-254890 depsipeptide

List of Figures

Appendix

This section of the thesis includes the full-length papers (only main texts), published, and submitted within the conduction time of the doctoral studies. The respective introductions to the papers are found in the chapters 2 – 6, in the same order: appendix A “Strategies towards Targeting Gαi/s Proteins: Scanning of Protein-Protein Interaction Sites to Overcome Inaccessibility”, followed by appendix B “Fluorescence Anisotropy Assay with Guanine Nucleotides Provides Access to Functional Analysis of Gαi1 Proteins”, appendix C “In-depth Characterization of Gαs Protein Activity by Probing Different Guanine Nucleotides”, appendix D “Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators”, and closing with appendix E “Bicyclic Peptide Library Screening for the Identification of Gαi Protein Modulators”. The copyright of the papers belongs to the respective publishers of the journals, as indicated by copyright statements displayed before each paper (when applied).

Appendix A: Strategies towards Targeting Gαi/s Proteins: Scanning of Protein-Protein Interaction Sites to Overcome Inaccessibility

B. Nubbemeyer, A. Pepanian, A. A. Paul George, and D. Imhof. Strategies towards Targeting Gαi/s Proteins: Scanning of Protein-Protein Interaction Sites to Overcome Inaccessibility. *ChemMedChem.* (**2021**) 16(11), 1697–1716, doi: 10.1002/cmdc.202100039.

ChemMedChem

Reviews
doi.org/10.1002/cmdc.202100039

Chemistry Europe
European Chemical Societies Publishing

Strategies towards Targeting Gαi/s Proteins: Scanning of Protein-Protein Interaction Sites To Overcome Inaccessibility

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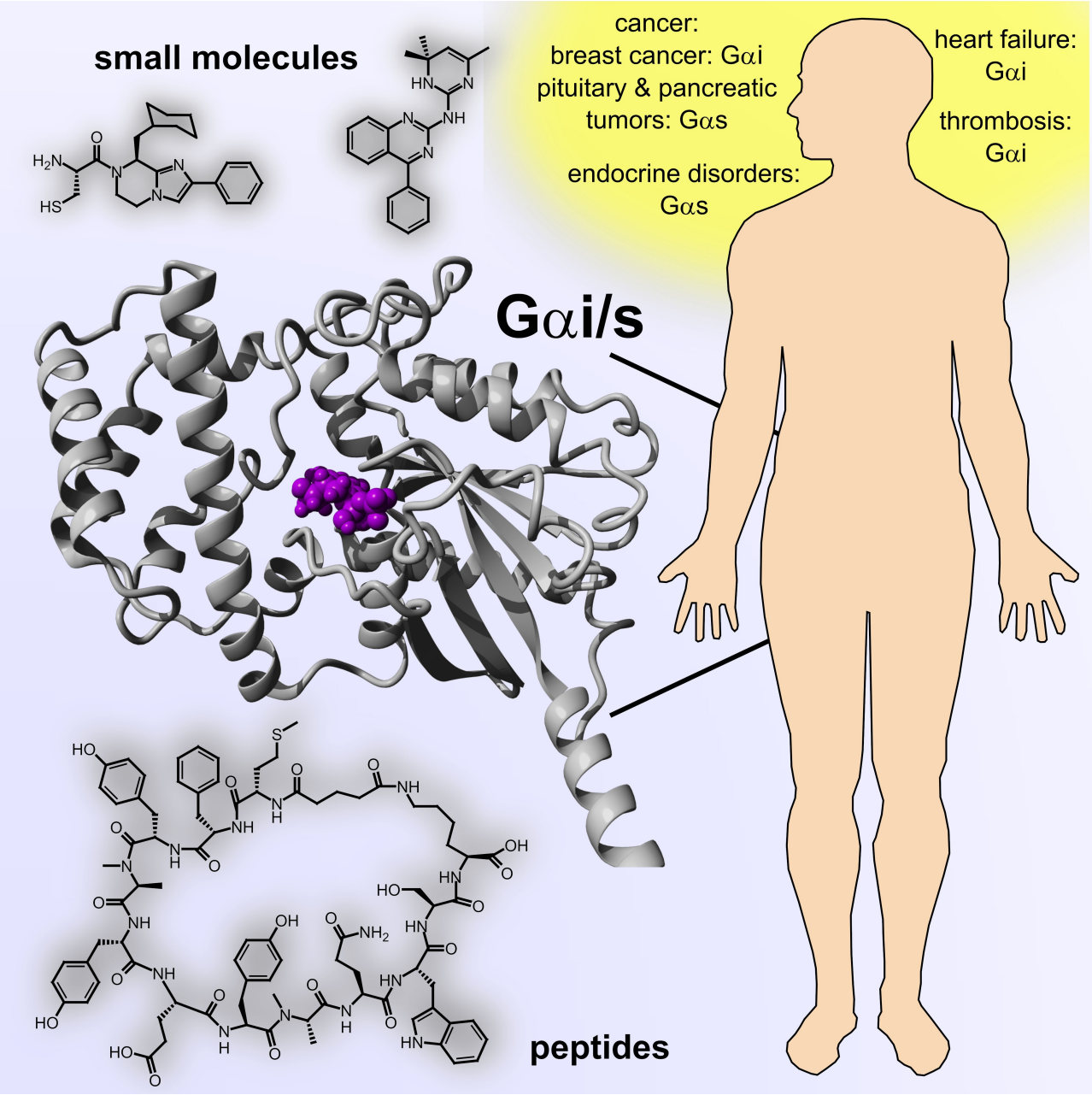

Heterotrimeric G proteins are classified into four subfamilies and play a key role in signal transduction. They transmit extracellular signals to intracellular effectors subsequent to the activation of G protein-coupled receptors (GPCRs), which are targeted by over 30% of FDA-approved drugs. However, addressing G proteins as drug targets represents a compelling alternative, for example, when G proteins act independently of the corresponding GPCRs, or in cases of complex multifunctional diseases, when a large number of different GPCRs are involved. In contrast to Gαq, efforts to target Gαi/s by suitable chemical compounds has not been successful so far. Here, a comprehensive analysis was conducted examining the most important interface regions of Gαi/s with its upstream and downstream interaction partners. By assigning the existing compounds and the performed approaches to the respective interfaces, the druggability of the individual interfaces was ranked to provide perspectives for selective targeting of Gαi/s in the future.

1. Introduction

G protein-coupled receptors (GPCRs) represent the largest family of transmembrane receptors with more than 800 members controlling the signal transduction of physiologically important processes. Through extracellular stimuli of the GPCRs, the signal is transmitted via membrane-bound, intracellularly localized heterotrimeric G proteins to intracellular effectors.[1–3] The indisputable importance of GPCR-mediated signal transduction is demonstrated by the fact that over 30% of the FDA-approved drugs target GPCRs (Figure 1A).[4,5] The attractiveness of addressing GPCRs lies in easily accessible druggable sites at the cell surface.[4,6] GPCRs are targeted for numerous diseases, including Alzheimer's disease and cancer. In particular, oncogenic mutations of GPCRs and G proteins have been identified in a significant number of tumors.[4,7–10] As randomly mutated GPCRs can occur, it is difficult to develop drugs that respond to each of these mutations. Furthermore, multiple GPCR signaling pathways may be involved in multifactorial diseases, such as asthma or cancer, making it unsuitable to address the GPCRs individually.[1,2,11] Therefore, targeting the downstream G proteins may be an appropriate alternative, further strengthened by the fact that overexpression, abnormal activation, mutations, and dysregulation of G proteins are attributed with diseases such as cancer (Figure 1B, C).[7,8,10] Besides cancer, G proteins are also associated with cardiovascular diseases, for example, heart failure, diabetes, and chronic inflammatory diseases like asthma.[1,12,13]

G proteins are often referred to as "undruggable" because they cannot be adequately targeted pharmacologically.[14] The intracellular location and the consequent lack of accessible sites on the cell surface is one of the reasons. Thus, molecules addressing G proteins need to pass the cell membrane to influence their activity. Of particular interest is the Gα subunit, which acts as a molecular switch by binding guanine nucleotide diphosphate (GDP, inactive) or guanine nucleotide triphosphate (GTP, active).

With respect to Gα, the four existing G protein subfamilies, Gαs, Gαi, Gαq/11, and Gα12/13 and their subtypes (Gαs: Gαs, Gαolf; Gαi: Gαi1-3, GαoA/B, Gαt1-2, Gαgust, Gαz; Gαq/11: Gαq, Gα11, Gα14-16; Gα12/13: Gα12, Gα13), have a high sequence and structural similarity, making it difficult to selectively address only one subfamily.[16–18] The development of selective and efficient G protein activators or inhibitors ("modulators") is of crucial importance, as they can be used as tools to gain deeper insights into G protein-mediated signaling and as lead structures to design therapeutic drugs. In this regard, various strategies have been applied to identify and develop modulators of G protein activity. For example, the investigation of natural compounds led to the discovery of G proteins in 1980, for which A. G. Gilman and M. Rodbell were awarded with the Nobel Prize for Physiology and Medicine in 1994.[19–21] Another possibility for the identification of G protein modulators are high-throughput screening techniques, which are commonly used to identify small molecules and peptides. Due to the structural similarity of the G protein subfamilies, small molecules might have only moderate target specificity, as can be exemplified with the imidazopyrazine derivatives BIM-46174 and BIM-46187.[22] Nevertheless, small molecules are able to interact with proteins specifically on protein "hot-spots".[23]

G proteins generally communicate through protein-protein interactions (PPIs) to regulate cellular processes.[24] In this context, the disruption of PPIs can lead to a specific modulation of the protein activity.[25,26] Thus, (macrocyclic) peptides are meanwhile regarded as suitable medium-sized molecules to interrupt PPIs, while the requirement for cell penetration can be met by incorporation of cell-penetrating peptide (CPP) sequences, as demonstrated for Cyclorasin 9 A5, targeting the small G protein KRas.[25,27–31] Today, peptidic modulators can be identified by several methods, including (computational) structure-based design or combinatorial approaches.[32–35]

Concerning Gα proteins, only the Gαq subfamily can be addressed sufficiently by the two naturally occurring cyclic depsipeptides YM-254890 and FR900359, which selectively inhibit the Gαq-mediated signaling pathway and are widely used in pharmacological studies, such as in uveal melanoma or asthma research.[1,36–41] As modulators like FR900359 and YM-254890 are still missing for Gαi and Gαs, we examined the existing strategies and developments to provide a comprehen-

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202100039

sive analysis of Gαi/s as targets for chemical tools as well as their interface regions (to GPCRs, Gβγ, effectors, accessory proteins), which are crucial for respective signal transduction pathways. Thus, this review aims at establishing the essential prerequisite for the future development of highly specific and potent modulators and tools for the investigation of G proteins and their involvement in diseases.

2. Gαi/s Interfaces: Determinants of G Protein Signaling

For the development of Gαi/s modulators, it is essential to understand their different signaling determinants (Figure S1 in the Supporting Information). A ligand binding to a GPCR results in conformational changes of the GPCR and the associated G protein and thus the GDP dissociation from the Gα subunit. The resulting empty-pocket conformation has a very short lifetime due to the high GTP concentration within the cell, which facilitates rapid GTP binding to Gα.[42] The latter induces the dissociation of the heterotrimer into GTP-bound Gα and Gβγ, which can address different intracellular effectors (Figure S1).[16,17,42] The signaling is terminated by the intrinsic GTPase activity of Gα, which causes GTP hydrolysis to GDP and phosphate. Following reformation of the heterotrimer, the GDP-bound G protein is restored to its original inactive state.[16,17] Further accessory proteins such as AGS proteins (activators of G protein signaling) or RGS proteins (regulators of G protein signaling) can stimulate G protein signaling or accelerate its deactivation.[43,44] AGS or RGS proteins can act as 1) GDIs (guanine nucleotide dissociation inhibitors), which stabilize the inactive, GDP-bound state and thus inhibit the activation of G proteins,[45] 2) GEFs (guanine nucleotide exchange factors), which can accelerate the exchange of GDP by GTP,[45] 3) GEMs (guanine-nucleotide exchange modulators), which have a bifunctional activity (GDI or GEF) depending on the G protein substrate,[46] and 4) GAPs (GTPase accelerating proteins), which enhance GTP hydrolysis and thus terminate the Gα signaling (Figure S1).[45,47]

Concerning the intracellular effectors (Figure S1), the Gαs subfamily stimulates the membrane-bound adenylyl cyclase (AC), which catalyzes the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). On the contrary, the Gαi subfamily members Gαi1-3 and Gαz inhibit AC and consequently the formation of cAMP.[48] Subsequently, cAMP can stimulate various downstream signaling pathways. Furthermore, Gαt1-2 stimulates photoreceptor phosphodiesterase (PDE), Gαgust is thought to stimulate PDE activity and absence of Gαo was found to be associated with ion channels' regulation.[16,48,49]

In order to map out possible directions for future strategies of Gα protein-targeted compound design based on the proteins' interface regions, it is required to analyze the structures of Gαi/s in the different activation states and ligand-complexed forms. Several X-ray and NMR structural analyses were reported in the past decades,[16,50] starting from the crystal structure analysis of Gαt in the active, GTPγS (guanosine-5′-*O*-(γ-thio)triphosphate)-bound state (1993), and the inactive, GDP-bound state (1994).[51,52] The Gα subunit has a conserved protein fold consisting of two domains, the GTPase domain (or Ras-domain, six-stranded β-sheet motif (β1-6) surrounded by five helices (α1-5)), which is structurally homologous to small G proteins and elongation factors of the G protein superfamily,

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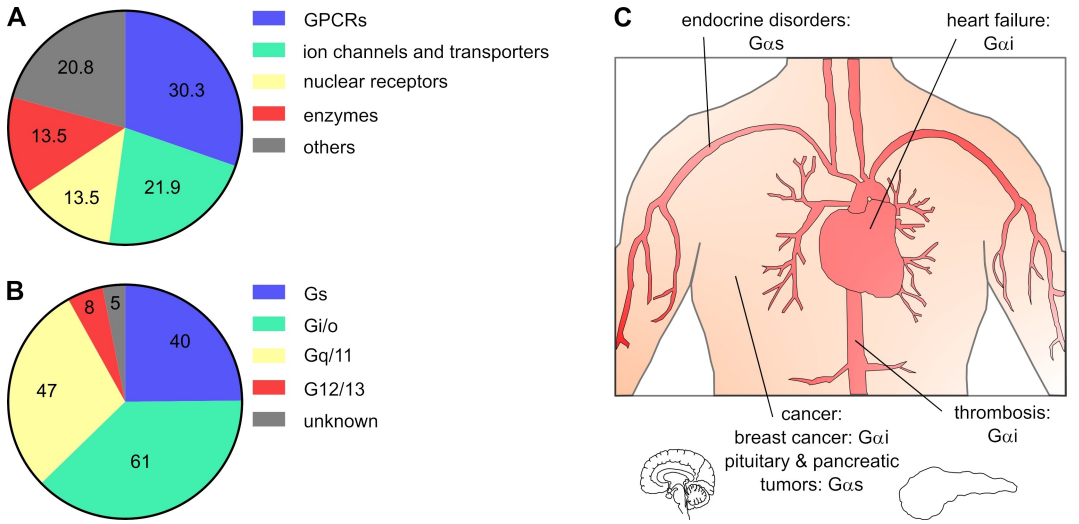

Figure 1. Involvement of GPCRs and G proteins in human diseases and drug development. A) Distribution of approved drugs (small molecules and biologics) per human protein family class derived from Santos et al.[15] B) Putative primary Gα protein coupling, based on the classification of GPCR signaling according to Sriram et al.[5] C) Involvement of Gαi/s subfamilies in multiple disorders such as cancer, heart failure, endocrine disorders or thrombosis, adapted from Li et al.[1]

and the helical domain (six α-helix bundle, with a large central helix (αA) surrounded by five smaller helices (αB–F)), which is unique for heterotrimeric G proteins (Figure S2).[51,52] Both domains are connected by two polypeptide segments, linker 1 and linker 2, resulting in the following sequence of structural elements starting from the N-terminal α-helix (αN): αN, β1, α1, linker 1, αA-F, linker 2, β2, β3, α2, β4, α3, β5, αG, α4, β6, α5.[51,52] Only the α3-β5 loop and the α4-β6 loop of Gαi1 and Gαs differ in their sequence and structural conformation within the conserved GTPase domain, which possibly influences the Gα binding to GPCRs and effectors.[53] The Gαi subfamily exhibits a high degree of conservation in sequence and structure, mostly distinguishable by minor differences in the helical domain.[53] In between the two domains is a deep cleft, where the respective guanine nucleotide is bound (Section 2.2).[51,52] Upon G protein activation, conformational changes occur in three adjacent regions, namely Switch I (linker 1, beginning of β2), Switch II (C-terminus of β3, α2, α2-β4 loop) and Switch III (β4-α3 loop, Figure S2), which are mainly located in the GTPase domain.[16,51,52] All Gα subunits, except Gαt, are reversibly post-translationally modified (PTM) with palmitate on a N-terminal cysteine.[16] Gαi subfamily members are additionally irreversibly myristoylated on an N-terminal glycine, which has a significant influence on αN. The latter is disordered in the unmodified state and gets ordered upon Gβγ binding, while the ordered αN in case of a myristoylated Gαi results in no further structural change during Gβγ binding. Furthermore, myristoylation might affect the effector interaction (Sections 2.4 and 3.4). Overall, PTMs are important for the regulation of membrane association and PPIs.[16,17,50]

The knowledge about the Gα structure supports the development of artificial modulators and the identification of natural products that influence the Gα protein activity. Therefore, it is helpful to know, that mostly the surface of the GTPase domain mediates interactions to GPCRs (Section 2.1), Gβγ (Section 2.3), downstream effectors (Section 2.4), and accessory proteins (Section 2.5, Figure 2).[50,53] The composition of the nucleotide binding pocket and the GTPase mechanism (Section 2.2) essentially contribute to the development of new Gα protein modulators.[44]

In the following, we describe the individual interface regions and their impact on the G protein-mediated signaling as well as the nature of the guanine nucleotide binding pocket in more detail. Our aim is to provide a more specific classification of the already known modulators (Section 3) by understanding the interface areas (Section 2), to assess the druggability of individual protein regions and thus to develop strategies for the identification of novel modulators.

2.1. Gαi/s-GPCR

For their pioneering work on GPCRs, Robert J. Lefkowitz and Brian K. Kobilka were awarded with the Nobel Prize in Chemistry in 2012,[56,57] which stresses the importance of G protein-mediated signaling. GPCRs are characterized by seven transmembrane-spanning α-helices (TM1-7), which are connected by three intracellular (ICL1-3) and three extracellular loops (ECL1-3). The N-terminus is extracellular and the C-terminus, which contains an α-helix (HX8) in class A GPCRs, is located intracellularly (Figure S3).[50] The TMs connect the extracellular ligand binding site with the intracellular binding site for the heterotrimeric G protein. Interestingly, the GPCR-G protein interface is about 30 Å apart from the GDP binding pocket, thus allosteric conformational changes within the interface and Gα result in the receptor-mediated GDP release. During reorganization of the cytoplasmic GPCR region upon receptor activation, the rotation and large outward movement

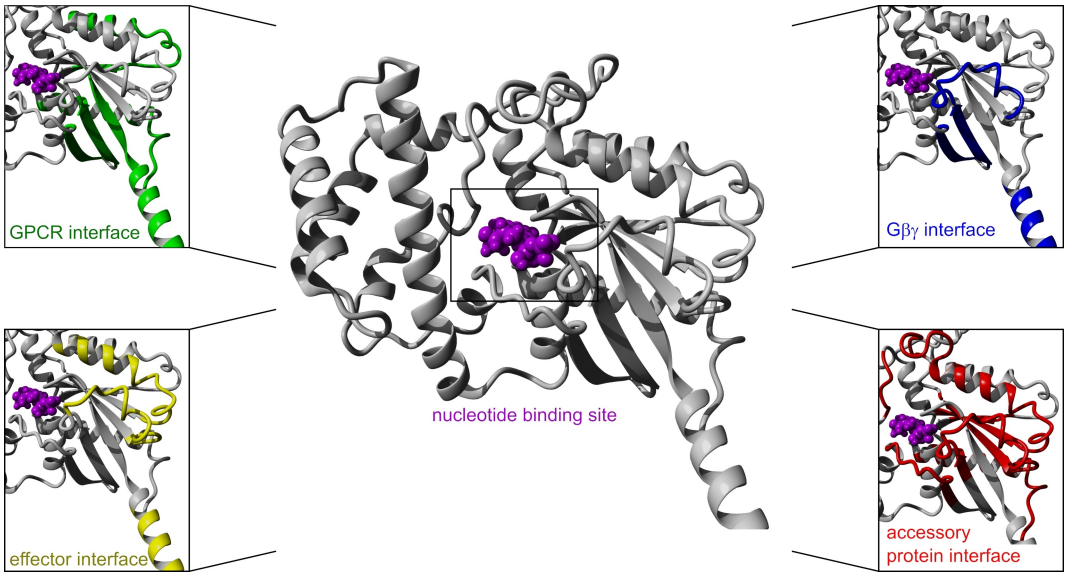

Figure 2. Structural features of Gα proteins: Contact areas to the GPCRs (green), Gβγ (blue), effectors (yellow) and accessory proteins (red, most common areas depicted) within the GDP-bound (violet) Gαi1 homology model (from PDB IDs: 3UMS[54] and 5JS8[55]).

of TM6 together with the rearrangements of TM1, TM4, TM5 and TM7 is characteristic.[58–60] This results in a cytoplasmic cavity, which can be occupied by the C-terminus of the Gα subunit, especially the "wavy hook" (distal C-terminus) and α5, after rotation and translation (Figure S3).[50,60–62] The resulting GPCR-Gα interface is formed predominantly by hydrophobic interactions between TM3, TM5-7, ICL3, HX8, and the Gα C-terminal part (α4, α4-β6 loop, β6, α5). A second, less extensive interface is established between αN, αN-β1 hinge, β1, β2-β3 loop, α5, and ICL2 (Figure S3). In addition, further Gα interactions (α3-β5 loop, α2, α2-β4 loop) with the GPCRs are described.[24,50,58,60,63]

Regarding the GPCR-G protein coupling selectivity, a significant difference between Gi- and Gs-GPCR complexes is the relative position of α5 (different rotation and orientation within Gαi/s) and TM6 (outward movement less intense for Gi- than for Gs-coupled GPCRs). This results in a wider open G protein binding pocket for Gs-coupled receptors and enables the binding of the sterically larger C-terminus of Gαs (α5 tilted up), whereas α5 of Gi binds relatively further down in the TM pocket allowing capping interactions with TM7/HX8.[58–64] Consequently, the Gα C-terminus is mainly responsible for the affinity and specificity of the G protein-GPCR interaction.[50,65,66] Beside α5, an impact of αN, the αN-β1 loop, the α4-β6 region, and α4 on the specificity of G protein coupling has been suggested, due to specificity determining residues within these regions.[24,50] Furthermore, TM6, ICL2 and ICL3 were related to mediate the coupling selectivity.[50,59,61,63]

2.2. Gαi/s-nucleotide

G proteins are called molecular switches, switching between the GDP-bound ("off") and the GTP-bound ("on") state to regulate the downstream signaling.[1,16] The determinants of nucleotide binding are based on the architecture of the binding pocket (Figure 3), which structurally alters within 1) GDP release and formation of the empty-pocket conformation, 2) GTP insertion and heterotrimer dissociation, 3) the GTPase reaction, and 4) the phosphate release together with the heterotrimer reassociation. In the following, the Common Gα Numbering system in the D.S.P. format (D: domain, with G: GTPase domain, H: helical domain; S: consensus secondary structure, with S: strand, H: helix; P: position within the secondary structure element, all in superscriptions) according to Flock et al.[67] is used to describe the involved Gα residues and to facilitate a comparison between the different Gα subtypes and subfamilies. Loops are written as lower case letters of the flanking secondary structure elements.[67]

The guanine nucleotide binding pocket is located deep in the core of Gα between both domains (Figure 3).[51,52] The nucleoside contacts are formed by interactions with both domains, whereas the phosphate contacts are mainly established with linker 2 and the GTPase domain.[52,68] Two conserved motifs, the NKXD$^{G.S5.7\text{-}G.HG.2}$-motif and the TCA(T/V)DT$^{G.s6h5.1\text{-}G.H5.1}$ motif ("TCAT-motif") are crucial for the binding of the guanine base and the stabilization of GDP in the binding pocket.[16,69] The phosphate binding is mediated by the highly conserved P-loop, GXGESGKST$^{G.s1h1.1\text{-}G.H1.3}$, which connects β1 with α1. Furthermore, the RXXTXGI$^{G.hfs2.2\text{-}G.S2.1}$ motif and the DXXG$^{G.S3.7\text{-}G.s3h2.2}$ motif are important for Mg^{2+} binding, whereby the latter motif connects the Mg^{2+} binding site with Switch II.[16,67,69–72] Mg^{2+} is octahedrally surrounded by ligands and coordinated by four water molecules, Ser43$^{G.H1.2}$ (P-loop) as well as the β-phosphate in the inactive state.[51,52,73]

GDP release and formation of the empty-pocket conformation. For GDP dissociation, domain separation is required along with the destabilization of the GDP-binding contacts mediated by

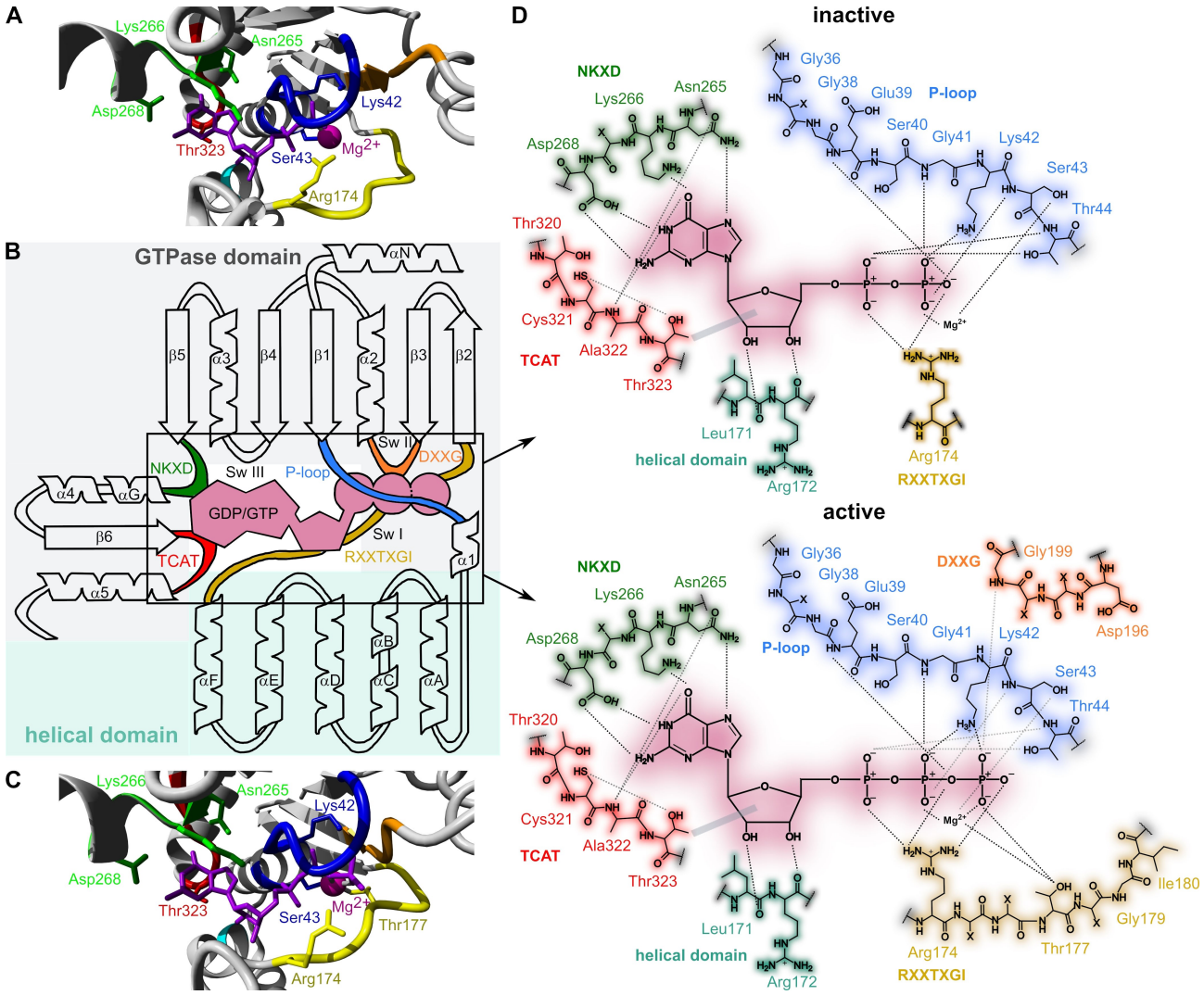

Figure 3. Contacts of Gα to bound nucleotides. Gαt crystal structures (GDP-bound: PDB ID: 1TAG[52] (A), GTPγS-bound: PDB ID: 1TND[51] (C), nucleotides in violet), domain arrangement[84] of Gα proteins (B) and contacts of nucleotides (D) to the P-loop (blue), RXXTXGI (yellow), DXXG (orange), NKXD (green), TCAT (red), helical domain (cyan) are shown. Dotted lines indicate hydrogen bonds and grey bars van der Waals interactions. Residues are named according to the crystal structures.

GPCR-induced conformational changes inside the G protein.[58,72,74–77] The conformational changes in α5 cause structural rearrangements of the adjacent β6-α5 loop (contains TCAT motif, Figure 3) and the reduction of hydrophobic interactions between α5 and α1, β2, and β3, and thus a destabilization and structural change of α1 (contains P-loop, Figure 3). As a consequence, the interface between the helical domain and the GTPase domain is disrupted and the GDP affinity is reduced.[58,76,78–80] However, the reduced contacts of α5 with β1-3 are compensated by new interactions to β4-β6, which stabilize the receptor-bound complex.[80] Beyond that, the αN-β1-loop contributes significantly to GDP dissociation by disturbing P-loop contacts to GDP.[17,58,72,76] The GDP release is favored as a result of the reduced GDP contacts along with a higher structural dynamic in the nucleotide-binding region.[58,72] In the resulting ternary complex, the helical domain exhibits increased dynamics and moves away from the GTPase domain.[76] In addition, the structure of the nucleotide binding pocket, especially the β6-α5 loop, is more dynamic and exhibits a larger solvent-accessible surface area, which promotes fast GTP binding induced by the high intracellular GTP concentration.[81]

GTP binding and dissociation of the heterotrimer. GTP binding leads to stabilization of α1 and the interdomain interface and induces the reclosure of both domains to a more rigid Gα structure.[55,63,76,80] Herein, Mg^{2+} and GTP are deeply buried in the binding pocket due to rearrangements of Switch I ($Arg174^{G.hfs2.2}$, $Thr177^{G.hfs2.5}$, RXXTXGI motif), Switch II ($Gly199^{G.s3h2.2}$ and α2), and Switch III (Figure 3A, C, F).[69] The structural changes within Switch I are induced by hydrogen bond formation between the γ-phosphate of GTP with $Thr177^{G.hfs2.5}$ and $Arg174^{G.hfs2.2}$, and the replacement of two water ligands on Mg^{2+} by $Thr177^{G.hfs2.5}$ and the γ-phosphate.[52,68] The conformational change of Switch I towards the Mg^{2+} binding site causes the interruption of Gα-Gβγ interactions and thus

contributes to the dissociation of the heterotrimer. The structural changes in Switch I and Switch II are connected through a newly formed hydrogen bond network.[52,68] Rearrangements in Switch II are initiated by a hydrogen bond formation between Gly199$^{G.s3h2.2}$ and the γ-phosphate of GTP, which is coupled to conformational changes of α2 conveyed by a hydrogen bond of Gly198$^{G.s3h2.1}$ with Trp207$^{G.H2.7}$. During this process, contacts of the conserved Arg201$^{G.H2.1}$, Arg204$^{G.H2.4}$ (ion pairs with Glu241$^{G.H3.4}$, Switch III) and Trp207$^{G.H2.7}$ to conserved residues in α3 are formed.[52,68] Switch III (e.g., Glu232$^{G.s4h3.10}$, Glu241$^{G.H3.4}$) responds to the conformational changes of Switch II by forming a network of polar interactions with Arg201$^{G.H2.1}$, Arg204$^{G.H2.4}$, and the Gly199$^{G.s3h2.2}$.[52,73] Additional residues within the β4-α3-loop and α3 stabilize the active conformation of Switch III through interaction with the helical domain.[73] The GTP binding leads to a destabilization of the heterotrimer, mainly by changes within Switch II, and initiates dissociation into Gα and Gβγ (Section 2.3).[73]

GTPase reaction. During GTP hydrolysis, the highly conserved Arg174$^{G.hfs2.2}$ ("arginine finger", Switch I, RXXTXGI motif) decisively stabilizes the pentavalent transition state by interacting with the β- and γ-phosphates of GTP (Figure 3D).[68,82] Additionally, the highly conserved Gln200$^{G.s3h2.3}$ (Switch II) is essential for the hydrolysis by interacting with the γ-phosphate and the nucleophilic water, which initiates the in-line attack on the γ-phosphate.[68,83] Hence, mutations of Arg174$^{G.hfs2.2}$ or Gln200$^{G.s3h2.3}$ have been observed in a number of human tumors, demonstrating the importance of these residues and the GTPase reaction for the G protein signaling.[82] Within the hydrolysis mechanism, the water molecule is further stabilized by the Thr177$^{G.hfs2.5}$.[68–70,83] RGS proteins are able to accelerate the GTPase activity (Section 2.5).

Dissociation of phosphate and heterotrimer reassociation. In the resulting Gα·GDP·Pi complex, Switch I moves marginally away from the catalytic site leading to a weaker Mg^{2+} binding and a hydrogen bond formation of Arg174$^{G.hfs2.2}$ with the β-phosphate and Pi, as well as Thr177$^{G.hfs2.5}$ and Lys176$^{G.hfs2.4}$. Switch II undergoes a significant structural change, which breaks the ionic interactions with Switch III, resulting in a disordered Switch III. Thereby, Gln200$^{G.s3h2.3}$ is shifted away from the active center, a transient phosphate binding site is formed and the Pi release is enabled.[83] The latter results in disordered parts of the Switch II and thus, Switch I shifts away from the nucleotide binding site, whereby Lys176$^{G.hfs2.4}$ rotates out of the active center, along with Mg^{2+} and Thr177$^{G.hfs2.5}$. Then, Arg174$^{G.hfs2.2}$ is only weakly associated with the α- and β-phosphate.[83] As Switch II is crucial for effector recruitment and Gβγ binding (Section 2.3, 2.4), the structural changes in Switch II reduce the affinity towards the effectors and promote Gβγ binding.[73] The binding of Gβγ rearranged Switch II and, furthermore, the conformational changes within Switch I and Switch II seal the GDP in the nucleotide binding pocket.[83]

2.3. Gαi/s-Gβγ

Gβγ is composed of two polypeptide chains, Gβ and Gγ, which can only be separated under denaturing conditions.[18,85] Crystal structure analyses revealed that Gβ exhibits an N-terminal α-helix and a seven bladed propeller structure composed of seven WD40 sequence repeats with four twisted β-strands per propeller blade (Figure S4). Gγ comprises two α-helices, with the N-terminal helix binding to the N-terminal helix of Gβ via coiled-coil interactions and the C-terminal helix engages with the propeller. The membrane association is controlled by prenylation of the Gγ C-terminus.[85–88] The contacts between Gα and Gβγ are primarily made via two interface regions between Gα and Gβ (Figure S4). The first interface is established between the top of the Gβ propeller by hydrophobic interactions with the hydrophobic pocket of Gα formed by Switch I and Switch II (especially β2, β3, β3-α2 loop, α2, Figure S4). This interface is additionally stabilized by hydrophilic/ionic interactions. The second interface is located between blade 1 of the Gβ propeller and αN of Gα. There is no structural evidence for direct interactions of Gα and Gγ.[53,85–88] The structure of Gα in the heterotrimer differs from free Gα.[86,87] In the heterotrimer, the αN helix is continuous, whereas in the free state the N-terminus can exhibit various structures.[86,87] The myristoylation of the N-terminus increases the affinity of Gα to Gβγ (Section 2).[89] The GTP-induced conformational changes especially in Switch II (Section 2.2) lead to the heterotrimer dissociation by interruption of the stabilizing contacts within the first interface.[85–88]

2.4. Gαi/s-effector proteins

Crystal structure experiments of Gα-effector complexes showed that the effectors insert hydrophobic side chains into a pocket formed by the N-terminus of α2 (Switch II) and α3. The effector specificity is defined by contacts with the C-termini of α2 and α3 as well as interactions with the α2-β4 loop and the α3-β5 loop.[16,49,53,90–92] Since the α3-β5 loop differs in sequence and structure between the subfamilies, it was assumed that it plays the key role in effector selectivity.[49,53] A further contribution of the α4-β6 loop was also reported.[16,53,90,93]

The Gαi and Gαs subfamily can interact with different effectors, however, both subfamilies have an opposite effect on the AC, whereby Gαs can bind to and activate all membrane-bound isoforms of AC (ACI-IX) and Gαi1 and the near paralogs can only address certain AC isoforms (ACI, V, VI).[90,94–96] The AC consists of a cytosolic N-terminus, two transmembrane domains separated by the cytosolic domain C1 (C1a–b), and followed by a further cytosolic domain C2 (C2a–b, Figure S5). The active site is located in the interface between C1 and C2.[97] The Gαs-AC interface is established between Switch II (α2 and α2-β4 loop) by insertion of α2 into the groove of AC (formed by C2), and the α3-β5 loop with C1 and C2. At the same time, Phe991(C2) binds into the Switch II/α3 cleft.[91–93,95] Mutagenesis experiments and molecular docking studies indicate that the Gαi-AC interface is located between C1 and Switch I–III as well as αB, which is opposite to the Gαs binding site on AC (Figure S5). Thus the

binding of Gαs and Gαi to the AC is not competitive.[53,90,93,98] Further studies with Gαs and Gαt showed that the N-terminus is crucial for effector binding. In the Gαs subfamily, no PTM is necessary for the stimulatory function, whereas myristoylation of the Gαi subfamily is required for AC inhibition.[16,53,97,99,100]

After GTP hydrolysis, Gα dissociates from AC due to a lower affinity of the Gα·GDP compared to Gα·GTP. Although Gα·GDP still has the ability to interact with effectors, its potency is lower than that of Gα·GTP. Reassociation with Gβγ terminates effector signaling since the Gα binding site for Gβγ (inactive state, Section 2.3) largely overlaps with the effector binding site (active state).[16]

2.5. Gαi/s-accessory proteins

Accessory proteins are capable of interfering with the G protein signaling in different ways, in particular by binding to Gα (Figure S6) and thus modulating the Gα activity. AGS proteins are divided into classes I–IV with I) GEFs (all Gα subclasses), II) GDIs (Gαi-selective), III) Gβγ binders or IV) Gα16-specific.[43,101–103] RGS proteins are categorized into different structural and functional classes, which are named after the prototypical member (i.e. A/RZ (Gαz/i-specific), B/R4 (Gαi/o/q-specific), C/R7 (Gαi/o-specific), D/R12 (Gαi/o-specific)). Typically, such proteins act as GAPs preferably with the Gαi subfamily.[44,103,104] In the following, the structural aspects of 1) GDIs, 2) GEFs, 3) GEMs and 4) GAPs are described in more detail.

GDIs. GDIs comprise one to four GPR motifs (G protein regulating motif, TMGEEDFFDLLAKSQSKRMDDQRVDLAG,[105,106] also known as GoLoco motif, consensus XXΦΦXΩΩX[+]XQπXRΩXXQR,[107,108] Φ: hydrophobic, Ω: aromatic, π: small, X: any amino acid)). The GPR motifs bind to and stabilize Gαi·GDP, thereby inhibiting the nucleotide exchange and the accompanied G protein activation (Figure S6). GDIs can prevent the association of Gα with Gβγ through overlapping interface regions, which may lead to prolonged Gβγ signaling.[45,103,108,109] The binding of the GPR motif is directed to Switch II/α3, where Arg of the Asp/Glu–Gln–Arg triad of the GPR motif is oriented towards the GDP binding pocket and directly interacts with the α- and β-phosphate of GDP.[45] The insertion of Arg is enabled by the conformation of Gln (triad), which interacts with $Gln147^{H.hdhe.2}$ and $Asn149^{H.hdhe.4}$ of Gαi. The GPR motif also establishes contacts to Switch I and changes its conformation, for example, $Arg178^{G.hfs2.2}$ (RXXTXGI motif, Section 2.2) is displaced by a salt bridge with $Glu43^{G.s1h1.1}$ (P-loop) and forms contacts to the GDP ribose entity. Further conformational changes occur in Switch II and Switch III. The C-terminal part of the GPR motif binds along the interdomain region, thus possibly restricting interdomain movements and preventing GDP dissociation.[102,108–111] Gαi specificity is assumed to be mediated by contacts with the helical domain (αA-αB loop, αB-αC loop),[102,108–111] and/or an acidic residue in the GTPase domain that influences the orientation of $Glu43^{G.s1h1.1}$.[112]

GEFs. The chaperones for nucleotide-free Gα subunits Ric8 A (resistance to inhibitors of cholinesterase, Gαi/q/12/13-specific) and Ric8b (Gαs/olf-specific) also function as GEFs through partial Gα unfolding (in absence of Gβγ).[43,113,114] They bind preferentially to Gα·GDP, cause GDP dissociation by domain separation and stabilize the empty pocket conformation, although GTP binding leads to Ric8 dissociation due to a lower binding affinity (Figure S6).[114,115] Three Gα contact sites for Ric8 proteins were referred: α5, β4–6 and Switch II/α3 together with the P-loop.[113,114,116] Similar to GPCRs, Ric8 interaction leads to a major structural changes of α5 and detachment from the hydrophobic β-sheet core (β4–6), which also rotates and is then stabilized by Ric8. The α5 movement disrupts the nucleotide contacts of the TCAT motif and the NKXD motif and destabilizes the purine binding site (Section 2.2). The antiparallel β2–β3 hairpin moves away from the GTPase core, which destabilizes and disordered α1 and thus leading to domain separation of Gα, destabilization of the P-loop contacts to GDP and enhanced GDP dissociation.[113,116–118] The interaction of Ric8 A probably shifts Switch II to the binding position of the γ-phosphate, which is associated with conformational changes in Switch I and promotes GTP binding.[116–118] The interruption of the contacts between Switch II and Ric8 A during GTP binding leads to the reorganization of β2 and β3, and Ric8 A dissociation. The selectivity determinants of Ric8 are probably family-specific residues of Gα (α5), whereby the majority of Ric8 A and Ric8B residues are conserved in the Gα contact region.[113,116–118]

GEMs. GEMs are the most recently discovered class of G protein-affecting proteins, with GIV (Gα-interacting, vesicle-associated protein) being first described as GEM (GEF for Gαi, GDI for Gαs).[46,119] GEMs possess a common motif (~30 amino acids, core consensus ΦTΦX[D/E]FΦ-motif,[120] Φ: hydrophobic, X: any amino acid) that selectively binds to the GDP-bound or empty-pocket conformation and affect monomeric Gα (Figure S6).[84,121] So far, only the GEF binding to Gαi3 has been structurally analyzed. The binding of the GEM motif to the cleft formed by Switch II (mainly contacts with $Gln204^{G.s3h2.3}$, $Trp211^{G.H2.7}$, $Phe215^{G.h2s4.1}$), α3 and the α3–β5 loop, induce conformational changes in Switch I (RXXTXGI motif), β1, and the P-loop and thus in the phosphate binding, which is sufficient for Gα activation.[84,121] Allosterically induced conformational change of the β2-β3 loop with associated α5 movement and disturbances in the interdomain interface (Switch III, αD–αE loop) is also observed, with the latter potentially resulting in domain separation.[84,121] The binding site of the GEM motif partially overlaps with the GDI and the Gβγ binding site.[84,121]

GAPs. GAPs interact with Gα·GTP and are able to catalyze GTP hydrolysis by stabilizing the transition state. The respective RGS proteins contain a functionally conserved RGS domain (~120 [B1] amino acids, "RGS box"), which is responsible for the Gα interaction and the catalytic activity.[45,103,122] The RGS domain forms an interface to Gα, recognizing and stabilizing mainly residues in Switch I-III (Figure S6). Three critical contacts are reported: 1) A hydrogen bond between Asn128 (RGS4) and $Gln204^{G.s3h2.3}$ (Switch II), which orients $Gln204^{G.s3h2.3}$ (Section 2.2) to stabilize the γ-phosphate and the nucleophilic water molecule. Asn128 also interacts with Switch II, thus stabilizing the conformation of Switch I and II. 2) A hydrogen bond between Asn88 (RGS4) and $Thr182^{G.hfs2.6}$ (Switch I), which brings Switch I–II into the conformation of the transition state, thereby

Thr182$^{G.hfs2.6}$ (Switch I) gets in contact with Lys210$^{G.H2.6}$ and Glu207$^{G.H2.3}$ (Switch II). 3) Asp163 (RGS4) stabilizes Thr182$^{G.hfs2.6}$ (Switch I), allowing the adjacent Thr181$^{G.hfs2.5}$ (Switch I) to stabilize the Mg^{2+} and to bring the nucleophilic water into an ideal position for GTP hydrolysis.[44,91,104,122,123] RGS contacts with Switch III and the helical domain (αA, αB-αC loop) are differently pronounced in the subtypes of the Gαi subfamily and possibly contribute to Gα selectivity and the potency of GAP activity.[91,104,122,124–127] The binding side of RGS proteins is consistent with the fact that RGS proteins are antagonists for effectors.[122,127] The specificity of the Gαi subfamily compared to the RGS-GAP incompetent Gαs subfamily can be explained by differences in the primary structure of the switch regions.[91,104,122,124,125]

3. Modulators Targeting Gαi/s Interfaces

The analysis of the Gα interface regions demonstrates that the contact regions are predominantly located in the GTPase domain (especially Switch I–III, β-sheet core, α3, N- and C-terminus). The helical domain is crucial for the nucleotide exchange and may serve as a specificity feature within the Gα subfamilies, as Gαi subfamily members are mostly distinguishable by minor differences in the helical domain.[53] The analysis also reveal which regions are exposed at the Gα surface and can be targeted by potential modulators. For example, Switch II/α3 may be regarded as "druggable" because it is addressed by Gβγ (Section 2.3), effectors (Section 2.4), and accessory proteins (Section 2.5). The latter show that binding to this region may have a functional impact on Gα and therefore represents an interesting model for modulator development (Section 3.5). Additionally, α5 (important for G protein activation, allosteric connection to nucleotide binding pocket), and αN (important in GPCR coupling, Gβγ binding and PTMs), are also interesting target structures (Section 3.1, 3.3). In the following, the individual interfaces are investigated for already known Gα binders and/or modulators as well as their identification methods. The classification of the individual interfaces according to their druggability provides important perspectives for future modulator development.

3.1. Gαi/s-GPCR

Within the Gα-GPCR interface, the C-terminus (wavy hock, α5) and the N-terminus (αN, αN-β1, β1) play significant roles in the allosterically induced GDP release (Figure S3). The essential function of the C-terminus for the GPCR coupling as well as its selectivity was recognized very early. For this reason, antibodies targeting the C-terminus of the Gα subunit were developed (Supporting Text in the Supporting Information, Figure S10).

3.1.1. Natural compounds

A number of natural compounds have been described for the Gα-GPCR interface. These include a bacterial exotoxin and numerous cationic amphiphilic substances, such as venom peptides from bees or wasps, whereby the latters can reversibly influence the Gα protein activity (Figure 4).

Pertussis toxin (PTX, 105 kDa[128]), is an exotoxin from *Bordetella pertussis* and inhibits the Gαi subfamily (except Gαz, Figure 4A, B). It can exert a mono-ADP-ribosyl transferase activity, covalently and irreversibly transferring an ADP-ribose element from nicotinamide adenine dinucleotide (NAD^+) to the C-terminal Cys$^{G.H5.23}$ conserved in the Gαi subfamily. Consequently, Gi uncouples from the receptor, cannot be activated, and remains GDP-bound leading to cAMP accumulation and various pathological effects in the host cell.[1,21,128–130] In addition, G protein-independent actions have also been described, which renders PTX together with its irreversible modification incapable for clinical use. Nevertheless, PTX has been applied in numerous studies to analyze Gαi-specific effects.[1,129,131,132]

A variety of cationic, amphiphilic substances, including neuropeptides, hormones, venom peptides, and polyamines, exhibited activating properties on purified G proteins. They have a high proportion of hydrophobic and basic groups orienting in an amphipathic α-helical structure in the presence of phospholipids (Figure 4C), and allowing them to penetrate the cell membrane.[134,135] Prominent members of this group are

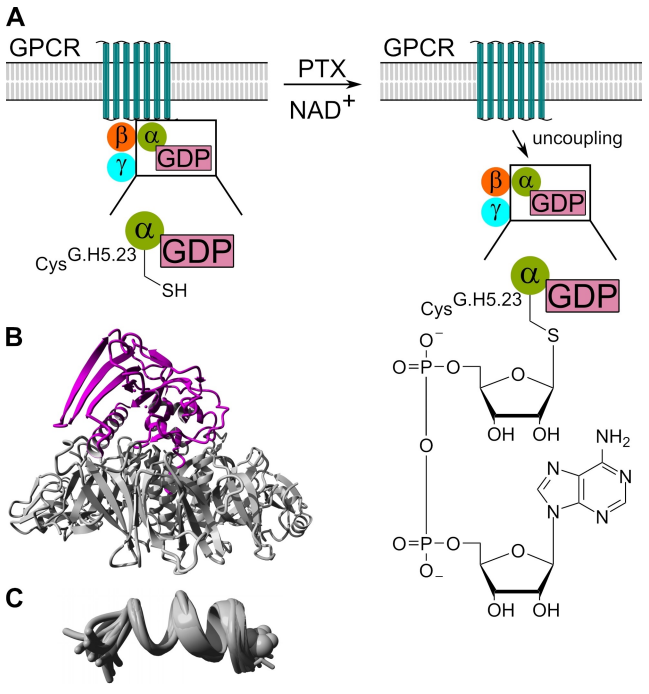

Figure 4. Natural compounds targeting the Gα-GPCR interface. A) Modification of Gαi by pertussis toxin (PTX) derived from Mangmool et al.[129] PTX transfers the ADP-ribose element from nicotinamide adenine dinucleotide (NAD^+) to Gαi Cys$^{G.H5.23}$. B) Crystal structure of PTX (gray, PDB ID: 1PRT[128]). The S1 subunit (magenta) is important for Gαi inhibition. C) G protein-bound NMR structure ensemble (14 structures) of mastoparan-X (H-INWKGIAA-MAKKLL-NH_2, PDB ID: 1 A13[133]).

the wasp venom 14mer peptide mastoparan (H-INLKALAALAK-KIL-NH_2) and the bee venom 26mer peptide melittin (H-GIGA-VLKVLTTGLPALISWIKRKRQQ-NH_2). Both venom toxins are able to disrupt cell membrane phospholipids and to cause lysis.[131,136–139]

Mastoparan and related analogs (mastoparans) increase the rate of GTP binding in a GEF-like manner and the GTPase activity for Gi/o, but have only a weak effect on Gt and Gs (except mastoparan-S, H-INWKGIASM-α-aminoisobutyryl-RQVL-NH_2).[131,133,134,136,140,141] Mastoparan has been shown to engage the Gα N- and the C-terminus and competes with GPCRs for G protein binding and thus has been used as low-molecular-weight GPCR mimetic.[133,142–147] Melittin comprises a predominantly hydrophobic N-terminus and a hydrophilic C-terminus. It stimulates Gi activity and inhibits Gs activity, which consequently leads to inhibition of AC activity.[139,148,149] Furthermore, activating effects on G proteins and their GTPase activity were reported for the neurokinin substance P (H-RPKPQQFFGLM-NH_2), synthetic polyamine component 48/80 (C48/80, mixed polymer of *p*-methoxy-*N*-methyl phenylethylamine crosslinked by formaldehyde), the mast cell degranulating peptide (H-IKCNCKRHVIKPHICRKICGKN-NH_2, MCD), and other cationic amphiphilic substances.[132,134,136,142,150–157] Altogether, these compounds are considered as pharmacological tools and candidates with potential therapeutic applications.[137,158] In the context of Gα modulators, the broad use of compounds such as melittin and mastoparan, is restrictive because of their dose- and cell-type dependency, nonspecific targeting and thereby induction of various biochemical effects.[159,160]

In summary, the natural compounds interact mainly via the Gα C-terminus, which appears well exposed and druggable, and thus cause GPCR-G protein uncoupling. For PTX, this results in a permanent inhibition of Gi, whereas the cationic amphiphilic peptides lead to GPCR-independent activation and signaling. The latter is a valuable starting point for tool development at the G protein level, which circumvents the need to address many GPCRs in multifactorial diseases.

3.1.2. Synthetic compounds

The described modulators from natural sources revealed that cationic hydrophobic substances are able to act as G protein modulators. Thus, these compounds have been further investigated. One synthetic compound is the polyamine C48/80 (Section 3.1.1), which activates Gi/o and stimulates GTPase activity.[141,142] In addition, other cationic hydrophilic substances such as hydrophobic amines[136,157] or derivatives of the lead mastoparan[136,138,161] have also been described as Gα modulators.

Quaternary hydrophobic amines have been referred in the context of mastoparan and can affect the activity of purified recombinant G proteins. For example, benzalkonium chloride (BAC) antagonizes the Gi stimulation of mastoparan by inhibiting the GDP exchange, whereas BAC alone slightly increases the basal GDP exchange at high concentrations. In contrast, BAC and other quaternary amines has been suggested to stimulate the nucleotide exchange and the GTPase activity of Go in response to the phospholipid concentration.[136] Other quaternary long-chain alkylamines displayed equally stimulatory properties on Go, whereas short-chain amines were ineffective. However, high concentrations of hydrophobic amines destabilize the G protein and might lead to denaturation.[136,157] Overall, these amines are considered unsuitable for the modulation of Gα protein activity, since they may also bind unselectively to other proteins and influence their activity.

In numerous studies, various derivatives of mastoparan (synthetic and natural) were investigated to explore the structural determinants, including net charge, spacing, charge localization, and proportion of α-helical conformation (Figure 4C), which define activity and cytotoxicity of the lead.[136,138,147,161,162] To reduce the cytotoxicity of mastoparan towards mammalian cells, [I^5, R^8]-MP was developed by replacing Ala5Ile and Ala8Arg, resulting in antimicrobial activity against bacteria and fungi but no cytotoxicity in HEK293 cells or hemolytic effects towards human erythrocytes.[138] Consequently, mastoparan is a prototype substance for the derivation of valuable antiinfective agents from naturally occurring antimicrobial peptides. However, due to G protein-independent side effects, these compounds are less attractive as G protein modulators.[138] In addition to mastoparans, GPCR-derived peptides have been extensively studied in order to gain insight into G protein-GPCR coupling and coupling selectivity.[163–166] These GPCR-derived peptides, however, have a comparably low potential, since each peptide can only interfere with the G protein signaling of a few receptors possessing, for example, similar ICL regions.

In summary, although the Gα-GPCR interface appears to be druggable, the existing modulators for this interface have many drawbacks for application as tool compounds. The interface might not be well suited for selective Gα targeting, due to the fact that there are multiple GPCRs adressing the same Gα subfamily. Thus, the selective modulation of one distinct Gα protein within the Gα-GPCR interface requires different modulators to affect one G protein signaling cascade entirely. Apart from this, this interface shows potential for exploiting the different coupling selectivities of a GPCR to a Gα protein to selectively affect a special GPCR-Gα interaction. In this context, however, it appears easier to address the extracellular druggable sites of a GPCR.

3.2. Gαi/s-nucleotide

The nucleotide binding pocket is not a typical PPI interface like the other regions described, wherein, different guanine nucleotides (GNPs, Figure S7) are able to bind. As GNPs are not classical modulators and can bind unspecific to other guanine nucleotide-binding proteins, we will only briefly discuss them here. More detailed information can be found in the supporting information. One application of GNPs is the ability to induce different activity states, as demonstrated by various crystal structure experiments and studies for quantifying the percentage of active G protein.[51,52,68,167,168] Altogether, GNPs represent

crucial tools for the analysis of G protein-affecting compounds, as they can be used, for example, in radioactive or fluorescently labeled form, to determine the impact of the tested compound on the nucleotide exchange as well as on the GTPase activity.[167,169,170] Consequently, GNPs proofed to be efficient for various applications.[51,52,68,167–170]

3.3. Gαi/s-Gβγ

There are not many modulators that address the Gα–Gβγ interface by approaching Gα, thus we decide not to subdivide this section. As shown in Section 2.3, Gα contacts Gβγ on the switch regions and αN (Figure S4).[86] The G protein activation enables the heterotrimer dissociation, whereby upon reassociation, the signaling is terminated since the effectors and Gβγ share Gα binding sites (Section 2.3, 2.4).[87,171,172] Furthermore, AGS class II proteins, such as AGS3 (contains four GPR motifs, Section 2.5), are able to dissociate the heterotrimer, since the GPR motif attaches and changes the conformation of Switch II close to the Gα-Gβγ interface. Consequently, modulators identified or developed for the Gα-accessory protein interface may also affect the Gα-Gβγ interaction (Sections 2.5, 3.5, Figure S6).[109,173–175] Moreover, Gβγ seems to compete with the fluorescently labeled Alexa532-RGS4 protein for binding with high affinity to Gαi·GDP·AlF4$^-$, which implies that Gβγ can inhibit the action of GAPs by binding to Gα.[176] Apart from that, the prenylation of Gγ (Section 2.3) anchors Gβγ in the plasma membrane and is highly required for the interaction with Gα and effectors.[177–179]

Based on the G protein signaling partners, peptides that bind to Gα on the Gα-Gβγ interface were developed. Kimple et al.[109] exploited the RGS14 GoLoco region to design R14GL (DIEGLVELLNRVQSSGAHDQRGLLRKEDLVLPEFLQ) derived from rat RGS14 (also accessory protein interface), that binds to Gαi between Switch II and α3 but not to Gαo, whereas the interaction with Switch II imbricates the contact of Gαi1·GDP and Gβγ.[109] Subsequently, Wang et al.[182] developed a Gβ-derived peptide exhibiting the respective Gαi1-binding sequence of a second Gβγ binding site on Gα, which was able to interrupt the respective Gαi1·GDP-Gβγ association.[182]

In addition to the natural partners within G protein signaling, researchers intended to study PPIs by targeting the Gα-Gβγ interface via different screening approaches. In this regard, Gβγ modulators have also been developed, however, are not described herein.[85,180] Suramin (**1**, Figure 5) is a drug discovered by Bayer in 1916 and used to treat the African sleeping disease. Initial studies implied that suramin binds directly to Gαs, hinders the heterotrimer reassociation and thus the G protein–receptor coupling.[1,183,184] Afterwards, experiments revealed that suramin inhibits the GDP release from Gα. However, suramin exhibits reduced selectivity, since it can inhibit Gαi and Gαs.[1] Consequently, different suramin analogs have been developed such as NF449 (**2**) and NF503 (**3**, Figure 5), which were superior to the other, comprising a higher selectivity for Gαi and Gαs.[1,2,181,183,185–187] The structural basis and the pharmacological importance of these agents needs to be more specified in the future. A further suramin derivative (NF023, Figure 7, Section 3.5.2.1.) was identified to target the Gαi3-GIV binding site.[188] A major drawback of these compounds is their limited cell penetration due to the high negative charge of the sulfonic acid groups, thus decreasing their pharmacological potential.[2]

Based on the aforementioned reports, it can be concluded that this interface overlaps with the Gα-effector and -accessory protein interface which hamper a clear distinction. Thus, these common sites might be valuable targets for future therapeutic applications.[180,189]

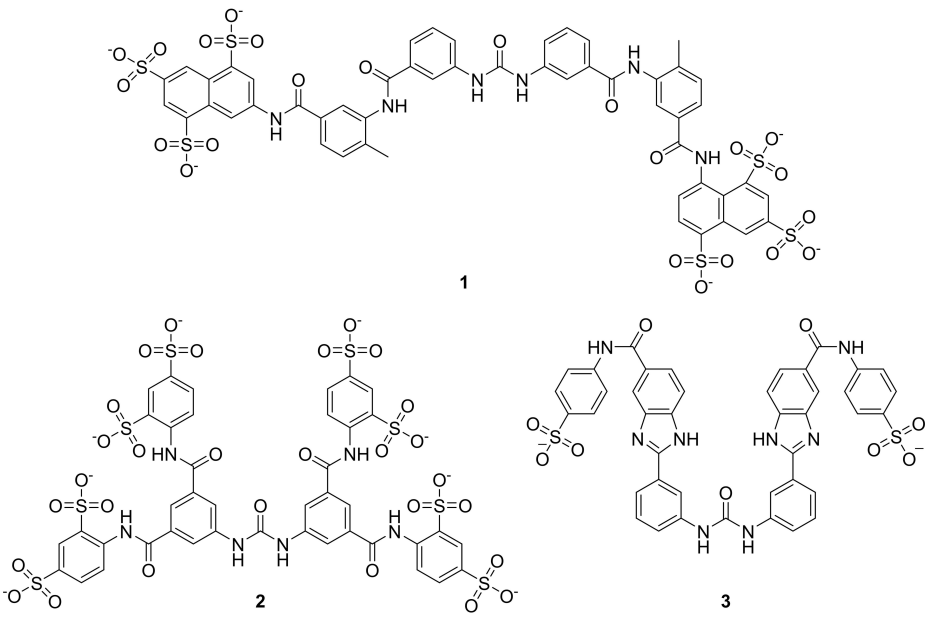

Figure 5. Chemical structures of suramin (**1**) and its analogues: NF449 (**2**) and NF503 (**3**).[1,180,181]

3.4. Gαi/s-effector proteins

Effectors of Gα are enzymes, proteins or ion channels with AC belonging to the most important effectors, which can be affected by Gαi and Gαs (Section 2.4, Figure S5).[48,53,190,191] As already mentioned, Gαi myristoylation is required for its inhibitory effects to distinct AC isoforms.[99] These findings provide a precious opportunity to modulate the Gα protein activity with PTM-like modifications. Apart from that, natural molecules that impair the association of Gαi/s and their downstream effectors are rare. Only accessory proteins, such as RGS16 (Section 2.5), can be given here since they may act antagonistically with respect to G protein-effector binding. In this regard, RGS16 was shown to bind to Gαt/o·GDP·AlF_4^- affecting the Gαt/o signaling pathway by inactivating the G protein-effector binding.[104,192,193] Based on these observations, the discovery of natural compounds or PTMs is anticipated to broaden the knowledge about this interface.

Likewise, there are only few examples of synthetic compounds that address this interface, which is why we have not divided this section further. It was already known in the 1970s that forskolin (Fsk) activates AC in a receptor-independent way.[93,190] What is striking though, is the contribution of the Fsk-Gαs·GTPγS complex in raising the binding affinity to two AC analogs, VC1 (ACV) and IIC2 (ACII) and their catalytic activity (Figure S8).[93] Furthermore, Yoo et al.[194] constructed AC-derived peptides and found that a peptide encoding C2-α′2 (899–926), and two more peptides, namely C1-β4-β5-α4 and C2-α3′-β4′, possessed inhibitory features regarding Gαs stimulation on full length ACII and ACVI (69% inhibition for the C1-peptide and 89% for the C2-peptides). Despite the aforementioned peptides, additionally tested peptides exhibited higher IC_{50} values, whereas others showed no inhibition.[194]

In summary, although crystal structures have provided insights into the Gαi/s effector binding,[90,93] the availability of compounds acting on this interface is rather low.[194] A possible explanation could be that the Gα-effector interface is not easy, if not impossible, to be manipulated. On the other hand, this interface overlaps partially with the interface for accessory proteins (Section 2.5, 3.5), making it non-trivial to clearly separate these regions. In our opinion, this interface may not be the most critical in studying G protein modulators, however, should not be neglected.

3.5. Gαi/s-accessory proteins

Accessory proteins themselves are modulators of Gα protein activity, acting as GDI, GEF, GEM, or GAP (Section 2.5, Figure S6).[45,46] Therefore, they serve as important templates for modulator development based on the motifs that are critical for their function and the interface that they bind to. Addressing the Gα-accessory protein interface and the GTPase activity, respectively, was of enormous importance in the past, as inhibition of the Gαs GTPase function by cholera toxin (CTX, Section 3.5.1) led to the discovery of G proteins.[21] Nowadays, accessory proteins have also been considered as drug targets, which is described in numerous excellent reviews.[44,103,195–197]

3.5.1. Natural Compounds

Regarding natural compounds targeting the Gα-accessory protein interface, it is important to consider that Gβγ (inactive state) and effectors (active state) represent natural competitors for the binding of accessory proteins, since the interface within Gα overlaps significantly (Section 2.3, 2.4, 3.2, 3.4).[16,45,49,109,121,122,127] Furthermore, bacterial exotoxins directly affect the GTP hydrolysis.[198] Cholera toxin (CTX, 84 kDa,[199] Figure 6A, C) is an exotoxin from *Vibrio cholerae*, the bacterium responsible for the symptoms of the cholera disease.[21] In early studies, it was observed that CTX increased the intracellular cAMP level by a permanent Gαs activation, leading to the discovery of G proteins.[21] The activation was caused by a mono-ADP-ribosyl-transferase activity of CTX (similar to PTX, Section 3.1.1), irreversibly transferring an ADP-ribose element from NAD^+ to $Arg201^{G.hfs2.2}$ (arginine finger, Section 2.2) of Gαs (Figure 6A).[1,21,193,198,200–202] As a consequence, the GTPase activity is inhibited and Gαs·GTP is prevented from being inactivated.[202–205] Using a similar mechanism, a heat-labile enterotoxin (HLT, 86 kDa,[206] Figure 6C) from *Escherichia coli* also selectively modifies and permanently activates Gαs.[1,201,206,207] Furthermore, a toxin from *Pasteurella multocida* (PMT, 146 kDa,[208] Figure 6B–C) modulates the Gα protein activity of Gαi/q/13. PMT catalyzes the deamidation of $Gln205^{G.s3h2.3}$ (Gαi) and conversion to $Glu205^{G.s3h2.3}$, thereby blocking the GTP hydrolysis (Section 2.2, Figure 6B). Consequently, Gαi remains in the active state resulting in a decrease in cAMP level.[1,82,209–211] PMT preferentially interacts, unlike PTX (Section 3.1.1), with monomeric Gα and can prevent conversion with PTX by Gαi deamidation.[211] In addition, *Photorhabdus asymbiotica* protein toxin (PaTox, 335 kDa, UniProt: C7BKP9, Figure 6C) causes the $Gln205^{G.s3h2.3}$ (Gαi) deamidation of Gαi/q/11 analogous to PMT and is also capable of catalyzing tyrosine glycosylation of Rho.[212] However, all of these bacterial exotoxins have the disadvantage to unrecoverably modify Gα, thereby irreversibly affecting the G protein activity. Therefore, these modulators have less clinical utility and should rather be regarded as important pharmacological tools that can provide insights into immunological processes or different aspects of G protein signaling.[201] However, it cannot be denied that targeting the GTPase function is a reasonable approach for modulating the Gα activity, since an inhibition maintains the Gα subunit in the active state whereas stimulation accelerates the termination of the signaling pathway.

3.5.2. Synthetic compounds

The enormous potential of the Gα-accessory protein interface has been recognized with the result that the development of novel tool compounds (small molecules and peptides) was primarily directed towards this interface region. High-through-

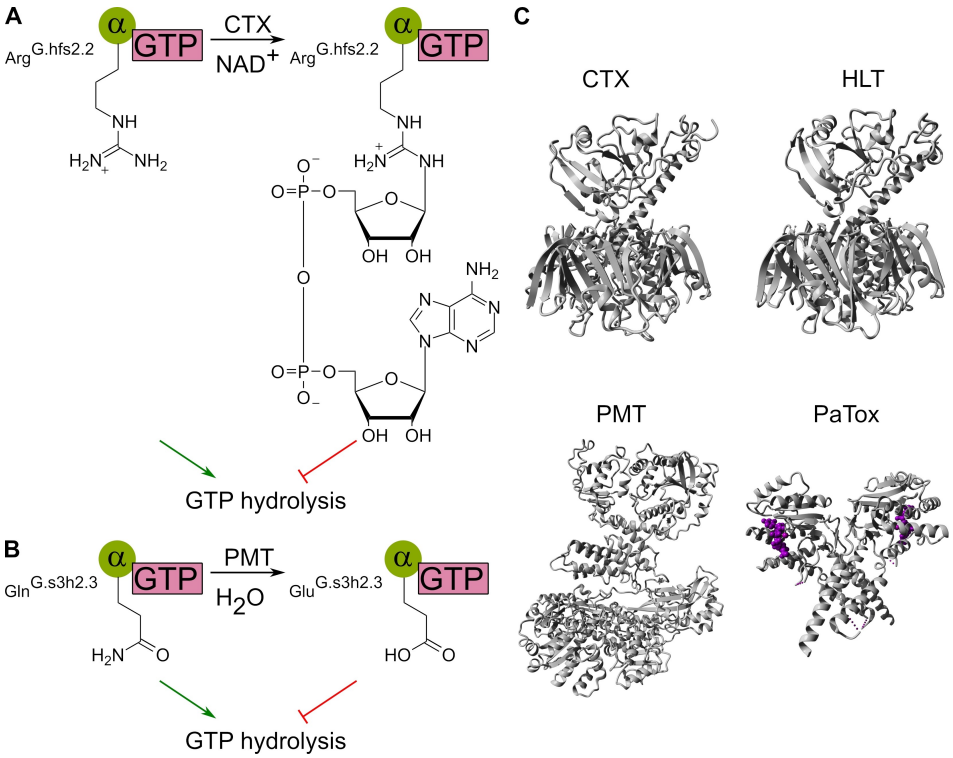

Figure 6. Natural compounds targeting the Gα-accessory protein interface. A) Modification of Gαs by cholera toxin (CTX). CTX transfers the ADP-ribose element from nicotinamide adenine dinucleotide (NAD^+) to $Arg^{G.hfs2.2}$ of Gαs, thereby inhibiting GTP hydrolysis. B) Modification of Gαi by *P. multocida* toxin (PMT). PMT catalyzes the deamidation of $Gln^{G.s3h2.3}$ to $Glu^{G.s3h2.3}$ and thus inhibits GTP hydrolysis. C) Crystal structures (gray) of cholera toxin (CTX, PDB ID: 1XTC[213]), heat-labile enterotoxin (HLT, PDB ID: 1LTS[207]), *P. multocida* toxin (PMT, PDB ID: 2EC5[214]) and the *P. asymbiotica* protein toxin (PaTox) glycosyltransferase domain (PDB ID: 4MIX[212]) in complex with UDP-GlcNAc (violet).

put techniques, but also virtual design, have been increasingly applied to identify or design novel modulators. Structure-activity relationships derived from crystal structures of complexes or molecular modeling and docking were frequently performed, too.[188,215–217]

3.5.2.1. Small molecules

The development of small molecule modulators is a classical approach in medicinal chemistry. In 2006 and 2009, the imidazopyrazine derivatives BIM-46174 (BIM-monomer, **4**) and the disulfide-bonded BIM-dimer BIM-46187 (**5**, both in short: BIM, Figure 7) were introduced, which showed antiproliferative and pain relief effects, respectively, and thus have been proposed as potential anticancer drugs.[11,218–220] For the selection of G protein-directed modulators, a differential screening approach with human cancer MCF-7 cells was applied, comparing the influence of potential modulators on CTX-stimulated cAMP production (Gαs-mediated signaling) with the influence on Fsk-stimulated AC activity (Section 3.4).[218] Both compounds act as pan-inhibitors of Gα protein activity, preferentially silencing Gαq signaling in a cellular context-dependent manner.[22,220] At the molecular level, BIM reversibly binds to Gα·GDP and prevents GTP binding after GDP dissociation.[11,22,220] Consequently, Gα is pharmacologically frozen in the empty-pocket conformation.[22] Using docking experiments and all-atom molecular dynamics simulations, Switch II, Switch III, and the αB-αC loop were postulated as BIM binding regions, which could explain the BIM-mediated inhibition through conformational changes in the switch regions that are crucial for GTP binding as well as a restricted domain separation of helical domain and GTPase domain.[11,22] In further studies, BIM was further analyzed with respect to Gαq targeting due to the Gαq preference.[221,222]

In a computer-based approach performed in 2014, molecular docking was applied to identify potential small molecules with GDI activity that bind to and stabilize Gαi·GDP in the presence of Gαi·GTP, Gαq·GDP, and Gαq·GTP.[223] Two compounds (0990 (**6**) and 4630 (**7**); Figure 7) with GDI selectivity for Gαi1 over Gαq, three compounds (8005, 8770, 4799) with GDI selectivity for Gαq over Gαi1, and three compounds (2967, 6715, and 1026) with GDI activity towards Gαi1 and Gαq were identified.[11,223] Some of these compounds were able to partially block the α2-adrenergic receptor-mediated cAMP regulation promoted by Gαi/o activation, however, neither compound showed the desired inhibitory activity even at high concentrations.[1,223] The quinazoline derivative 0990 was studied in more detail and was suggested to bind to Gαi·GDP ($Arg178^{G.hfs2.2}$/$Val199^{G.S3.6}$ or $Glu43^{G.s1h1.1}$/$Gln79^{H.HA.14}$ or $Gln79^{H.HA.14}$/$Lys180^{G.hfs2.4}$), all mimicking important Gαi1-GDI interactions. In structure-activity relationship studies, the basic hydrophobic

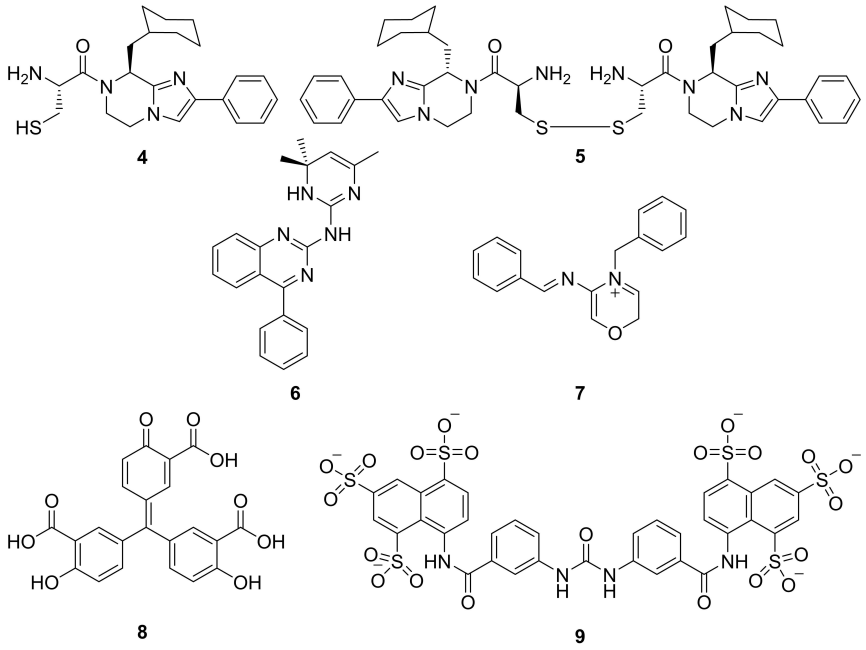

Figure 7. Chemical structures of small molecules targeting the Gα-accessory protein interface. Imidazopyrazine derivatives BIM-46174 (**4**) and BIM-46187 (**5**),[11] compounds 0990 (**6**) and 4630 (**7**),[223] aurintricarboxylic acid (ATA, **8**) and suramin derivative NF023 (**9**).[188]

phenyl-quinazoline-aniline core was shown to be crucial for the GDI activity.[11,223]

In 2017, by an *in silico* ligand screening and a separate high-throughput screening, the Gαi3-GIV interface (Section 2.5) was addressed, and NF023 (**9**, suramin derivative, Section 3.3) and ATA (**8**, aurintricarboxylic acid, both Figure 7) were identified. Both compounds were confirmed as Gαi3 binder and inhibitor of Gαi3-GIV binding.[188] NF023 binds to Switch II, α3 and α3-β5 loop, a binding site that overlaps with the binding site of the GEM motif (Section 2.5).[84,120,121] However, no interference with Gαi3–Gβγ binding was observed, although the interface regions partially overlap (suggested for suramin, Section 3.3).[188]

The disadvantage of these small molecules is that NF023 (and suramin) are not cell permeable and can inhibit P2X receptors in addition to Gα subunits, and ATA can also address other targets such as topoisomerase II.[1,188] Apart from that, the authors concluded that the Gαi–GIV interface is defined and druggable and thus of interest for modulator design.[188]

The screening approaches employing small molecules demonstrate the possibility to develop Gα modulators. However, a clear drawback is the selectivity of the compounds for the individual subfamilies or G proteins themselves. This is exemplified with BIM, a pan-inhibitor for Gα protein activity, obtained from a screening experiment towards Gαs, while the approach from 2014 identified compounds with Gαi/q selectivity that did not exhibit the anticipated inhibitory activity. NF023 and ATA also address other targets besides Gα and are therefore not specific. Nevertheless, small molecules are important tools to study G protein signaling pathways and to explore the determinants for selectivity between the subfamilies.

3.5.2.2. Peptides

The approach of peptide engineering is of particular interest regarding the Gα-accessory protein interface. For example, peptide sequences derived from protein motifs, such as the GPR motif,[106,107] GEM motif,[84,120] and RGS domain,[104,122] which are important for the corresponding functions as GDI,[106–108] GEM[119,120] or GAP,[122] can serve as templates for the peptide design.[45,46]

GPR proteins and GPR-derived peptides were shown to act as GDIs for Gαi *in vitro*.[1,102,224,225] Subsequently, CPPs such as a hydrophobic K-FGF-derived peptide sequence (AAVALLPAVL-LALLA) or basic TAT-derived sequence (GRKKRRQRRRPP) were attached N-terminally to a GPR motif (H-TMGEEDFFDLLAKSQ-SKRMDQRVDLAK-NH_2) to increase the cell penetration of the GPR peptide.[223] The TAT-GPR construct maintained GDI activity and selectively blocked Gαi regulation of α2-adrenergic-mediated AC activity in HEK293 cells.[223] The TAT-GPR construct has therefore been proposed as a valuable pharmacological tool and potential therapeutics. The authors, however, have tended to consider the development of small molecule inhibitors (Section 3.5.2.1) due to the relatively large size of the construct (40mer peptide).[223] In a similar approach, a GIV-derived peptide (GIV-CT, 210 amino acids), containing the GEM motif and an SH2-like domain, was N-terminally coupled to a TAT-PTD (peptide transduction domain) sequence to increase cell permeability.[226] It has been shown that the construct can bind to Gi in a cellular context and activates it in a GEF-dependent manner.[226] Consequently, peptides derived from accessory protein motifs can affect the Gα protein activity and intracellular modulation can be achieved by CPP attachment. The drawback to the described constructs is that they are relatively

large as to be used as chemical tools (e.g., 40mer peptide or protein).

mRNA display approach. Along with using the actual protein motifs to develop modulators, they have also been used as templates for high-throughput techniques (peptide sequences in Table S1). For example, the Roberts group used a GPR consensus-derived mRNA display library for the screening against Gαi1·GDP and identified the Gαi·GDP-specific R6A and minimized its sequence to the 9mer peptide R6A-1. Both peptides competed with Gβγ for Gαi1 binding. It was hypothesized that the GDI activity was conserved, however, this was contradicted in later studies for R6A-1.[227,228] R6A-1 binds to Switch II/α3 of Gαi1 and also showed binding to the other Gα subfamilies in the GDP-bound state.[228,229] Therefore, R6A-1 was postulated as a core motif for Gα interaction[227,229] and was subsequently used for the development of Gαi·GDP·AlF_4^- binders[230] and Gαs binders within Switch II/α3.[231] The first approach yielded AR6-05, which competes with Gβγ for Gαi1 binding and favors the GDP-bound more than the GDP·AlF_4^--bound state.[230] The second approach used a two-step selection process, identifying two Gαs·GDP-specific peptides (GSP), mGSP-1 and mGSP-2, which maintain specific contacts with Switch II/α3 and inhibit the formation of the heterotrimer. It was shown for GSP, mGSP-1, and mGSP-2 that they act as GDI for Gαs, with GSP also acting as GEF for Gαi1, thus showing bifunctional GEM-like properties.[231] Further optimization strategies of R6A-1 included N-methylations in order to increase its proteolytic stability.[232] By using an mRNA display with a macrocyclic peptide construct, the proteolytic stability towards chymotrypsin of the identified Gαi·GDP-selective cycGiBP (**10**, Figure 8) was significantly increased compared to its linear variant linGiBP. Both peptides compete with R6A for binding to Gαi1, and therefore an equal binding site was assumed.[233] Subsequently, the library was first digested with chymotrypsin, followed by mRNA display selection against Gαi1·GDP, leading to hits with increased chymotrypsin resistance and stability in human plasma.[234] The respective peptides were referred to as cyclic protease resistant peptides (cycPRP-1 (**11**), cycPRP-3 (**12**), both Figure 8). Due to the similar core consensus, it was suggested that both peptides also bind to Gαi1 on Switch II/α3.[234] By using an mRNA display containing also unnatural amino acids, the Gαi·GDP-selective SUPR (**13**, scanning unnatural protease resistant, Figure 8) was obtained exhibiting a further improved stability in human serum, a half-life of ~900 min in liver microsomes and a 35-fold better *in vivo* stability in mouse compared to cycGiBP.[235]

Recently, in a modified mRNA display approach, the Gαs·GTP-selective GsIN-1 (**14**, Figure 8) was identified using a Random nonstandard Peptide Integrated Discovery (RaPID) system, which also addresses Switch II/α3 and inhibits Gαs.[217]

Phage display approach. The first phage display towards Gαi1 was performed with a commercially available peptide library and two peptide families (consensus ΩPXXΩHP (peptide 1) and LPΩXXXH (peptide 3) with Ω: aromatic amino acids) with G protein-activating properties were identified, however, no structural information was described.[236] In another phage display experiment with Gαi1·GDP, the GDP-selective peptide KB-752 was discovered showing GEM-like activity (GEF for Gαi1 and GDI for Gαs) and high similarity to the GEM motif.[215,237] In a crystal structure analysis with Gαi·GDP, the peptide was shown to bind into the hydrophobic cleft of Switch II/α3 (like the GEM motif of GIV, Section 2.5, Figure S6).[215] Altogether, KB-752 is able to inhibit cAMP production through its bifunctional function within the G protein-mediated AC activity, which has been shown in cell membrane preparations.[237] In addition, a consensus to the previously described R6A-1 ([T/Y/F]-W-[WY]-[ED]-[FY]-L) was identified, based on which the Switch II/α3 binding site of R6A-1 and the subsequently developed mRNA display peptides were concluded.[228,231,233] In a second experiment, a phage display was performed with Gαi1·GTPγS, resulting in the active-state selective peptides KB-1753, KB-1746, and KB-1755.[216,238] KB-1753 is capable of inhibiting the interaction of Gαt with its effector cGMP PDEγ and Gαt-mediated activation of cGMP degradation, as well as interfering with RGS protein binding.[216,238] Crystal structure analysis of KB-1753 in complex with Gαi1·GDP·AlF_4^- showed that KB-1753 also binds into a conserved hydrophobic pocket between Switch II and α3.[216] Based on results in competition binding assays, it was shown that the Gαi1 binding sites of KB-1753 and KB-1755 as well as of KB-1755 and KB-1746 partially overlap, whereas the binding sites of KB-1753 and KB-1746 do not. Furthermore, KB-1755 was shown to interact with Gα the effector and RGS protein binding region. Thus, KB-1746 was thought to predominantly interact with the RGS binding site of Gα, as KB-1753 predominantly addresses the effector binding site.[216,238]

OBOC library screening. In a recent study, using an one-bead-one-compound (OBOC) library screening against Gαi1·GDP, we identified a peptide, GPM-1, with high sequence similarity to KB-752[237] and the GEM-motif,[119,120] which was further modified to increase cell permeability and proteolytic stability. The optimized peptides exhibited GDI activity towards Gαs and GEF activity towards Gαi1 in a GEM-like activity. Thus, the peptides may lower the cAMP concentration in the cellular context via the G protein-mediated AC activity. Using molecular modeling and docking analyses, the peptides were shown to bind to Gαi1·GDP similarly to KB-752 and the GIV-GEM motif within Switch II/α3. Such compounds may thus be considered valuable tools for the study of G protein-mediated signal transduction and pathogenesis (unpublished results).

In summary, the peptides described predominantly address the Switch II/α3 region (Figure S9), which appears to be well exposed and well targetable/druggable. This is demonstrated by the fact that this region is not only targeted in directed approaches, but also in non-directed attempts. The binding cleft between the Switch II α2-helix and α3 is well accessible within both, Gαi and Gαs, in either state of activity, as shown by the diverse peptides presented in this section. The variation in state selectivity and subfamily specificity is due to the varying conformation of the switch regions, which allows only peptides with certain structural features to bind. Thus, addressing the Switch II/α3 region is an interesting objective for future applications of both, peptides, which allow more selective binding due to larger interaction areas, and small molecules.

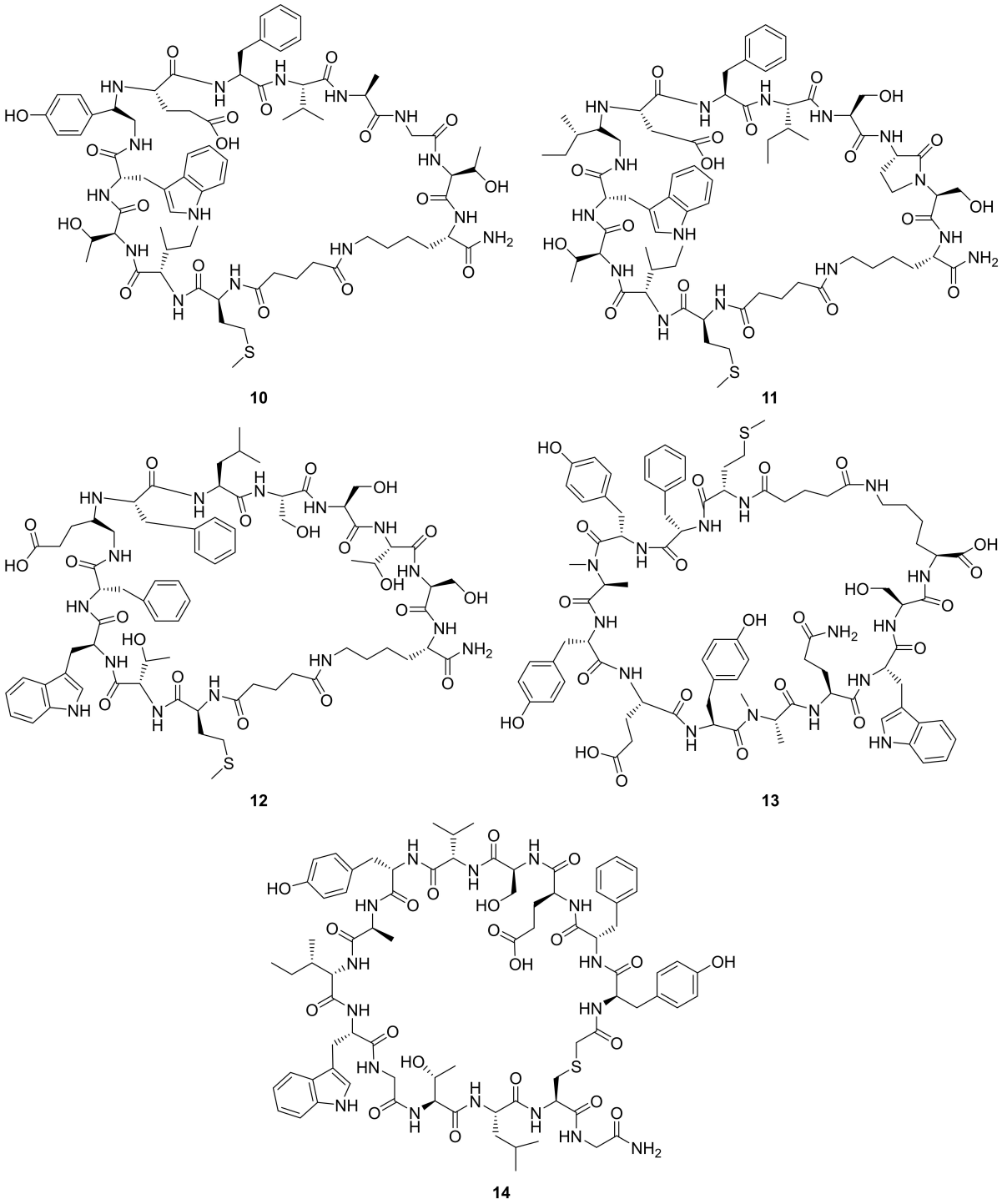

Figure 8. Chemical structures of mRNA display-derived peptides targeting the Gα accessory protein interface. The peptides cycGiBP (**10**),[233] cycPRP-1 (**11**), cycPRP-3 (**12**),[234] and Gα SUPR (**13**)[235] are Gαi1·GDP selective. GsNI-1 (**14**)[217] is Gαs·GTP selective.

4. Summary and Outlook

G proteins play a crucial role in signal transduction and in a variety of physiological processes. However, this might also indicate that G proteins are involved in the development and progression of diseases in case of malfunctions in respective signaling cascades. GPCRs are already targeted by over 30% of the FDA-approved drugs and are consequently well druggable through their extracellular ligand binding site.[4,5] However, targeting G proteins is an attractive alternative compared to GPCR-directed drugs, for example, in cases of multifactorial diseases, in which multiple GPCRs are involved, or in cases where the disease pathogenesis occurs downstream of the GPCR at the G protein level. To date, no drugs addressing G

proteins have been approved or tested in clinical trials, rendering the development of tool compounds crucial for pharmacological research.[1,2,11,180]

The Gα subunit of heterotrimeric G proteins has a high potential for manipulation by modulators, because of its various structural determinants and its role as molecular switch. Here, we examined the five different interaction sites of Gαi/s, namely the Gα-GPCR, the nucleotide binding pocket, the Gα-Gβγ, the Gα-effector, and the Gα-accessory protein interface, in more detail highlighting the structural characteristics of these interactions. Subsequently, all modulators known so far from the literature were assigned to one of these interface regions, and the approach used to identify these modulators was analyzed for its potential to provide an important starting point for targeting these previously "undruggable" proteins in the future.[14]

Regarding the Gα-GPCR interface, many natural compounds are known to address the Gα N- and C-termini, which are thus readily accessible to potential modulators, as evidenced for the N-terminus by its post-translational modifications and for the C-terminus by the ability to develop specific antibodies for this region (Supporting Information). However, the substances targeting this interface also exhibit non-G protein-specific activities, which renders them unsuitable for clinical studies and as leads. We consider this interface to be less attractive for modulator development, since the variety of GPCRs with their G protein coupling selectivities only allows to address few specific receptor-mediated signaling pathway simultaneously.

Targeting the nucleotide binding pocket by modulators is a suitable tool to study G protein signaling and to evaluate novel modulators occupying different interface regions. GNPs are important to induce artificially different activation states and thus distinct Gα conformations, for example within crystal structure analyses. Furthermore, GNPs are valuable in evaluating whether compounds affect the nucleotide exchange, and exhibit GDI, GEF or GEM activity, or alter the GTPase function, which might be achieved by binding of the respective compound to the Gαi/s-accessory protein interface. Additionally, GNPs are also critical for determining the quality of recombinant G proteins. For modulator development, these compounds are less suitable because they can also target other guanine nucleotide-binding proteins.

The assignment of modulators to the Gα-Gβγ and Gα-effector interface is not trivial, since the interaction regions overlap with the contact areas of accessory proteins, depending on the Gα activation state. Thus, these interface areas have potential for being addressed by tool compounds, although the development starting from the accessory proteins is more promising.

Finally, the Gα-accessory protein interface might possess the highest potential for modulator design, since accessory proteins themselves influence the Gα activity and can therefore be used as models or lead structures. This is evident from the fact that peptides derived from the GPR or GEM motif can affect the G protein activity *in vitro* or in conjugation with CPPs intracellularly. In addition to directed approaches that aimed to directly address this interface, non-directed high-throughput techniques also yielded compounds that were able to address this interface. These compounds were frequently associated with modulator properties. Overall, the analysis of this interface has shown that especially the Switch II/α3 region is well exposed and druggable, which has already been described by DiGiacomo et al.[188] in the context of small molecules, but can further be extended to the peptide level. This region could therefore be approached experimentally on the basis of protein motifs or already identified binders/modulators, or theoretically by directed docking experiments using the above-described approaches. Comparing the potential of small molecules with that of peptides indicates that peptides show a higher selectivity due to more specific contacts than small molecules. In addition, the identified peptide modulators of the Switch II/α3 region demonstrate that state-selective or subfamily-selective modulators can be developed, as the conformation of the Switch II/α3 binding cleft differs accordingly.

As a consequence for future investigations, novel modulators may be identified based on the conformation of the Switch II/α3 region, using especially directed high-throughput techniques, but also the already identified compounds, which can be further developed as lead structures. At the same time, the approach of identifying natural compounds should be considered as a valuable strategy, although it might be time-consuming and non-directed.

In conclusion, Gα proteins have an enormous potential for being targeted by pharmacological tools and drugs. Such compounds would provide a viable alternative to circumvent the necessity of targeting GPCRs in the future, especially in the context of multifactorial diseases or diseases associated with downstream defects of GPCR signaling.

Acknowledgements

Financial support by the University of Bonn, the Deutsche Forschungsgemeinschaft (FOR 2372, IM 97/14-1, to D.I.), and the Bonner Universitätsstiftung (to B.N.) is gratefully acknowledged. Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: G alpha proteins · peptides · protein-protein interactions · signal transduction · small molecules

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Manuscript received: January 15, 2021
Accepted manuscript online: February 22, 2021
Version of record online: March 22, 2021

Appendix B: Fluorescence Anisotropy Assay with Guanine Nucleotides Provides Access to Functional Analysis of Gαi1 Proteins

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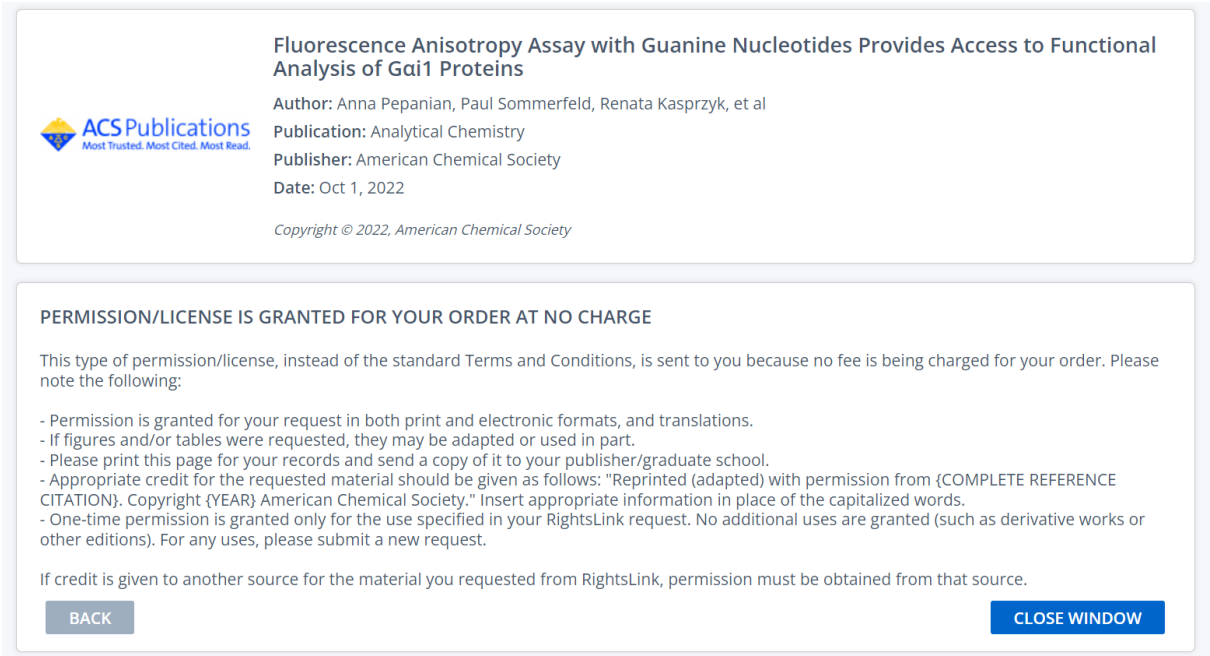

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Fluorescence Anisotropy Assay with Guanine Nucleotides Provides Access to Functional Analysis of Gαi1 Proteins

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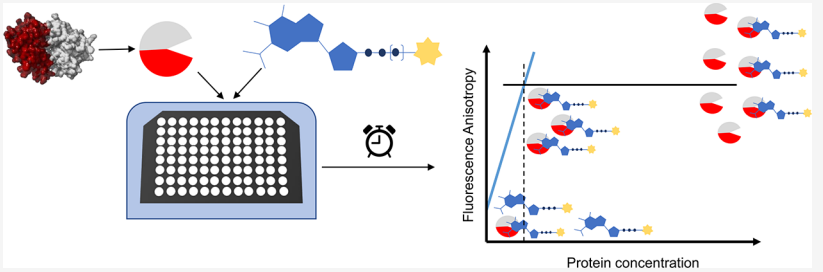

ABSTRACT: Gα proteins as part of heterotrimeric G proteins are molecular switches essential for G protein-coupled receptor-mediated intracellular signaling. The role of the Gα subunits has been examined for decades with various guanine nucleotides to elucidate the activation mechanism and Gα protein-dependent signal transduction. Several approaches describe fluorescent ligands mimicking the GTP function, yet lack the efficient estimation of the proteins' GTP binding activity and the fraction of active protein. Herein, we report the development of a reliable fluorescence anisotropy-based method to determine the affinity of ligands at the GTP-binding site and to quantify the fraction of active Gαi1 protein. An advanced bacterial expression protocol was applied to produce active human Gαi1 protein, whose GTP binding capability was determined with novel fluorescently labeled guanine nucleotides acting as high-affinity Gαi1 binders compared to the commonly used BODIPY FL GTPγS. This study thus contributes a new method for future investigations of the characterization of Gαi and other Gα protein subunits, exploring their corresponding signal transduction systems and potential for biomedical applications.

In all eukaryotic organisms, heterotrimeric guanine nucleotide binding proteins (G proteins, Gαβγ) can act as binary molecular switches in order to activate various intracellular signaling cascades upon activation of G protein-coupled receptors (GPCRs).[1,2] The Gα subunit is composed of an α-helical and a GTPase domain, and between the two domains lies the binding pocket for guanine nucleotides (Figure S1a).[3] The bound nucleotide, guanosine triphosphate (GTP, active conformation) or guanosine diphosphate (GDP, inactive conformation),[4,5] is responsible for the activity state and the downstream signaling effects. The binding affinity between the G protein and the nucleotide is highly dependent on phosphate and magnesium ion coordination upon the interaction of a phosphate-binding loop (P-loop) with the Switch regions I and II within the GTPase domain (Figure S1a).[3,6,7]

A plethora of studies exploring the purity, folding, and binding affinity of Gα proteins with several ligands (nucleotides and/or peptides) have been reported but lacked the final accurate determination of the protein's guanine nucleotide binding activity.[8−10] In fact, the activity of the expressed Gα proteins is commonly assessed by binding assays with various guanine nucleotideanalogues, for example, [^{35}S]-GTPγS as well as BODIPY- and MANT-labeled nucleotides,[9−13] via radioactivity- and fluorescence-based measurements and some indirect analyses, such as the trypsin protection and the GTPase assays.[8,14,15] It is noteworthy that radioligand binding assays have started to be substituted by more cost-effective and environmentally friendly concepts.[12,16−18] Examples of such alternatives are fluorescence anisotropy (FA) assays, which can be exploited to test Gα protein−ligand interactions.[17−19] As a ratiometric method, it is less sensitive to other fluorescent compounds interfering with binding assays.[20−22] Therefore, suitable fluorescently labeled ligands with low K_D values are desirable. In order to monitor the fluorescence changes, BODIPY- and MANT-based probes

Received: July 21, 2022
Accepted: September 22, 2022
Published: October 7, 2022

https://doi.org/10.1021/acs.analchem.2c03176
Anal. Chem. 2022, 94, 14410−14418

are commonly used, however, they lack broad applicability for FA-based assays. For instance, the spectral properties of MANT-labeled guanine nucleotides (excitation at 260/360 nm and emission at 440 nm)[11] restrict their broad use for FA-based assays, although they show varying ranges of binding affinities.[10,23] On the contrary, BODIPY-labeled probes have more red-shifted excitation and emission maxima but still exhibit K_D values in the high nM to low μM range, particularly toward Gαi proteins.[9,13] Therefore, analogues with favorable spectral properties that bind with high affinity are highly required. Since 1992,[24] the fluorophores 5-FAM and 6-FAM (5-/6-carboxyfluorescein) have been widely used for oligonucleotide binding (FA-based assays)[25,26] and, in general, for biopolymer binding studies.[27] Jarmoskaite et al.[20] successfully determined the total amount of active protein by assessing the proportion of bound radioligand to protein, leading to our assumption that FA experiments could be conducted for the quantitative determination of Gα protein activity, too.

In the present study, we outline an optimized and fast method to produce GTPase-active Gαi1 protein in high yields as well as a synthetic approach for new fluorescently labeled GDP/GTP analogues and their successful application in fluorescence- and FA-based binding assays. A scheme providing an overview of the methods used in this study and a work flow can be found in the Supporting Information (Scheme S1). Since Gαi1 expression was described in the literature to be less challenging compared to the other Gα family members,[8,28,29] it was initially selected for bacterial protein production and for investigating its GTP binding activity by the FA assay. We identified probes **17**−**20** as high-affinity binders of Gαi1 (K_D values in the one-digit nM range), verified by the microscale thermophoresis/temperature-related intensity change (MST/TRIC), with two of these probes (**17**, **19**) being applied in the quantification of the active fraction of self-produced and commercially obtained Gαi1 protein. GTPase activity of Gαi1 was additionally characterized by an optimized luminescence method (GTPase-Glo assay).[15]

The findings provided herein are fundamental for the implementation of further activity studies of the other Gα proteins by exploiting the advantageous properties of our probes and the FA-based method. In terms of the functional role of heterotrimeric G proteins, this approach will essentially support future investigations toward the understanding of the molecular basis of diseases and multifactorial disorders, such as cancer, associated with these proteins.

EXPERIMENTAL SECTION

Characterization of Active Gαi1 Protein Expressed in *Escherichia coli*. The gene of human (hexahistidine) His_6-tagged Gαi1 (Uniprot ID: P63096) was cloned into the pET28a (+) expression vector and transformed into *E. coli* BL21 (DE3) bacteria cells as previously described with slight modifications.[30] Bacteria were grown in LB medium (37 °C, OD_{600} of 0.4−0.6) and induction was initiated by the addition of 0.25 mM IPTG (4 h, 30 °C). Bacterial cells were lysed (1 mg mL^{-1} lysozyme and protease inhibitors), and the expressed protein was purified by Ni-NTA affinity chromatography and subsequently by size exclusion chromatography (SEC) on an Äkta Prime Plus device equipped with a HiLoad 16/600 Superdex gel filtration column. GDP (50 μM) was added throughout each purification step to avoid protein denaturation. The eluted protein fractions were tested for protein concentration and purity by the Bradford assay using ROTI-Nanoquant,[31] and SDS-PAGE, respectively, pooled together and stored at −80 °C. All protein aliquots were stored in elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM $MgCl_2$, 250 mM imidazole, pH 8.0) after the addition of 10% of glycerol. Prior to further experiments, the respective buffer exchange was carried out for Gαi1·GDP accordingly (Supporting Information).

Synthesis of Fluorescent Guanine Nucleotide Analogues. The synthesis and chemical characterization of compounds **2**, **4**, and **13**−**21** are described in the Supporting Information. The synthesis of compound **17** was performed by mixing an aqueous solution of **7** (triethylammonium salt;[32] 1.1 mg, 1.3 μmol, 0.14 M) with **10** (0.7 mg, 1.5 μmol, 0.05 M) in 30 μL of DMSO, followed by the addition of an aqueous solution of $CuSO_4{\cdot}5H_2O$ (0.75 mg, 3.0 μmol, 3.0 μL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 μmol, 6.0 μL, 1.0 M). The mixture was stirred at room temperature for 5 h. Then, the reaction was stopped by the addition of 400 μL of aqueous Na_2EDTA (7.4 mg, 20 μmol). The precipitate of unreacted fluorescein dye was removed by centrifugation (14,000 rpm, 10 min). The supernatant containing the reaction product was purified by semi-preparative reversed-phase high-pressure liquid chromatography (RP HPLC, method P3, Table S1). The collected eluate was repeatedly lyophilized to yield **17** ammonium salt as a yellow solid (0.2 mg, 0.2 μmol). HPLC conversion: 91%; yield after purification: 16%; HRMS (ESI^-) (m/z): $[M - 2H]^{2-}$ calcd for $C_{37}H_{34}N_9O_{19}P_3^{2-}$, 500.5598; found, 500.5592. For the synthesis of **19**, the same procedure was applied using **11** (0.9 mg, 1.8 μmol, 0.05 M) in 35 μL of DMSO instead of **10**. Probe **19** (ammonium salt) was obtained as a yellow solid (0.1 mg, 0.1 μmol). HPLC conversion: 51%; yield after purification: 7%; HRMS (ESI^-) (m/z): $[M - 2H]^{2-}$ calcd for $C_{39}H_{38}N_9O_{19}P_3^{2-}$, 514.5754; found, 514.5747. Further synthesis and analysis details are provided in the Supporting Information.

Fluorescence- and FA-Based Gαi1 Binding Assays. The investigation of the binding interactions between Gαi1 or recombinant human GNAI1 (enQuireBio, product no. QP12009, lot 1Z201229B) and probes **2**, **4**, and **13**−**21** was performed at 30 °C on a BioTek Synergy 2 multimode microplate reader with the software Gen 5 version 1.11.5. All experiments were performed in black, non-treated, 96-well F-bottom PS plates (FluoroNunc, product no. 237105, ThermoFisher Scientific) or black, non-binding, 384-well round-bottom low-volume PS plates (Corning, product no. 4514) at excitation and emission wavelengths of 485 nm and 528 nm, respectively. If not stated otherwise, total fluorescence intensities (Q) and both parallel and perpendicular fluorescence intensities ($I_\parallel$ and $I_\perp$, respectively) were measured directly after mixing the assay components (0 min) and after incubation of the assay mixture for 33, 66, 99, or 132 min. Values of FA (A) were calculated according to eq S3[33] from $I_\parallel$ and $I_\perp$ by means of the Gen 5 software, applying a G factor of 0.87 (pre-set value for correcting "the intrinsic bias of the detector system's response for one plane of polarized light over the other"[34]). A detailed description of reagents, assay performance (fluorescence intensity, FA, and competition assays), and data analysis with the corresponding equations utilized is available in the Supporting Information.

MicroScale Thermophoresis/Temperature-Related Intensity Change (MST/TRIC). MST/TRIC experiments were performed with a NanoTemper Monolith NT.115 Blue/Red (Blue mode) by mostly following the recommendations by

https://doi.org/10.1021/acs.analchem.2c03176
Anal. Chem. 2022, 94, 14410−14418

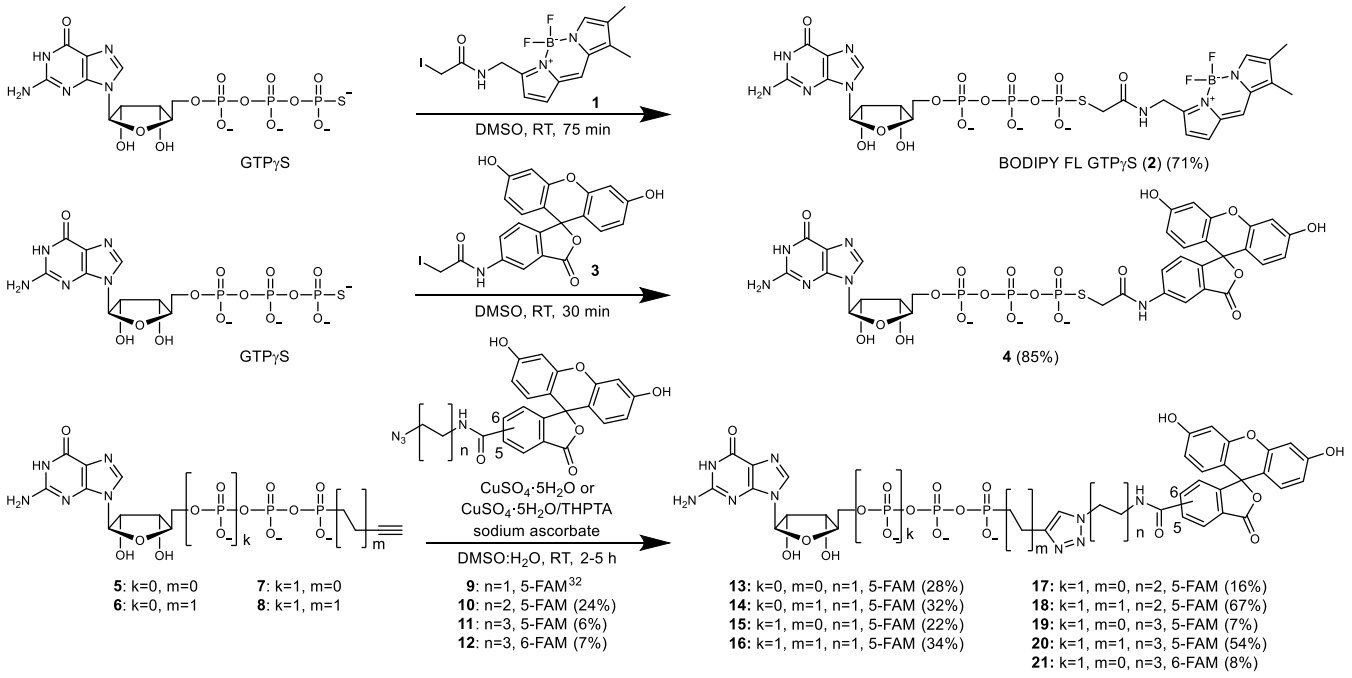

Figure 1. Synthesis of BODIPY FL GTPγS (**2**) and probe **4** by S-alkylation[50,51] of GTPγS with alkyl iodides **1** and **3**, respectively, and of fluorescein-containing probes **13**–**21** by copper-catalyzed azide–alkyne cycloaddition (CuAAC) reactions.[32] For CuAAc, alkynes **5**–**8**, representing the functional unit of the probes, were reacted with azides **9**–**12** carrying the fluorescent 5-FAM or 6-FAM moiety. Isolated yields after purification by RP HPLC are given in parentheses.

Sedivy[35] using probes **17**–**20** as fluorescently labeled components for direct comparison with results from the FA assay. Specific details of MST/TRIC experiments can be found in the Supporting Information.

GTPase Activity Assay. GTPase activities of lots 2 and 3 of active Gαi1 (amounts calculated by using the active fraction obtained with probe **19**) and recombinant human wild-type Ras expressed in *E. coli* (Merck, product no. 553325, lot 3497270) were determined on a BioTek Synergy 2 multimode microplate reader with the GTPase-Glo assay kit (Promega, product no. V7681, lot 0000475231) according to the manufacturer's protocol with several modifications as described in the Supporting Information.

RESULTS AND DISCUSSION

Production and Characterization of Gαi1 Protein Obtained from *E. coli*. In order to characterize the structure and function of the Gαi1 protein, the human recombinant His_6-tagged Gαi1 protein was expressed in *E. coli* BL21 (DE3) cells. The protocol used was inspired by earlier reports from several groups;[5,28,36] however, certain details were changed to optimize the quality and quantity of the protein. By keeping the induction temperature low (30 °C)[28,36] and the IPTG concentration higher (250 μM) as previously reported (30–150 μM),[10,36] the protein expression was induced 2.2–5-fold faster (4 h) compared to earlier reports.[28,36,37] Therefore, it was possible to express soluble Gαi1 in approximately 24 h, which is a time-beneficial approach compared to previous trials.[5,10,36,38]

The protein was purified in a two-step procedure, first by employing ion metal affinity chromatography using a Ni-NTA column and subsequently, by SEC (Figure S1b) in the presence of 50 μM GDP in each step to prevent protein denaturation, as has been earlier described.[5,10,39] Protein purity and identity were proven by a single band and a peak detected at ∼44.3 kDa via SDS-PAGE (Figure S1c) and MALDI-TOF mass spectrometry (MS, Figure S1d), respectively. As a control, a commercially available recombinant human His_6-tagged Gαi1 protein (enQuireBio, expressed in *E. coli*) was used for comparison (Figure S1c). The overall procedure for protein purification and recovery after expression resulted in 39.4–49.3 mg L^{-1} (bacteria culture) purified Gαi1·GDP protein in approximately 10 h. After the successful protein purification, CD analysis was conducted on Gαi1 to detect specific structural features, such as the main secondary structure elements of the protein. For this purpose, GDP was removed from the buffer to avoid signal interferences in CD measurements as observed with the GDP-containing buffer. All further experiments, including binding studies, were therefore performed in the absence of GDP. Previous CD-based structural studies on Gαi1 proteins have indicated a predominant α-helical (40–50%) and partial β-sheet character (9–11%),[8,10,38,40] which was supported by analysis of NMR and X-ray structures of Gαi1 bound complexes (homology model in Figure S1a).[10] The partial α-helical character of the Gαi1 was verified by the presence of a characteristic, strong maximum peak at 192 nm wavelength, accompanied by a double minimum at 208 and 222 nm (Figure S1e). The estimation of 41% α-helices and 11% β-sheets in the expressed protein by means of the K2D2 algorithm[41] is in good agreement with previously reported results as mentioned above.[10,40,42] Evidently, our expression and the two-step purification protocol provided large amounts of soluble Gαi1 protein of high purity and with the expected secondary structure. To our knowledge, the protocol introduced in this study is one of the fastest approaches reported so far for both prokaryotic[28,36] and eukaryotic expression systems,[5,43] leading to high yields (up to 9-fold higher[10,28,37,38] yields) of recombinant His-tagged human Gαi1·GDP when expressed

https://doi.org/10.1021/acs.analchem.2c03176
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in *E. coli*, without the formation of chimeras or the necessity of co-expression with accessory proteins.[36,44,45]

Synthesis of Fluorescent Guanine Nucleotide Derivatives as Probes for Gαi1. Up to date, various guanine nucleotide analogues have been used to test the activity states of Gα proteins based on their ability to exchange nucleotides.[3,11,46] In our recent review,[2] we reported that BODIPY- or MANT-labeled GTPγS derivatives have been extensively used for quantifying their binding affinity to the Gαi1 subfamily.[9,11] BODIPY FL GTPγS (**2**), in particular, has often been applied in fluorescence assays[9,11] and to a lesser extent in FA/fluorescence polarization-based approaches[19,47] on Gαi. However, this fluorescent probe slowly undergoes hydrolysis in the presence of Gα protein (which remains undetected when using fluorescence intensity as a readout but requires measurement of FA as proof[48]). We therefore aimed at the generation of advanced fluorescent guanine nucleotide analogues with increased binding affinity and hydrolytic stability as potent tools to address the GTP-binding side of Gαi1 by a FA assay. The fluorophore carboxyfluorescein (FAM) was selected for the labeling of GDP- and GTP-derived analogues since fluorescein dyes have properties beneficial for FA measurements, such as high quantum yields and relatively short fluorescence lifetimes of the excited state (3.8 ns), and are most commonly used for generating FA probes applied in high-throughput screening assays.[27] The functional part of the probes was based on GDP or GTP analogues equipped with an alkyne handle at the terminal phosphorus atom (Figure 1, Table S2), that is, **5**−**8**,[32,49] which enabled straightforward attachment of ω-azidoalkylamido derivatives of 5-FAM or 6-FAM (**9**−**12**, accessible via amide coupling with TSTU)[32] by applying conditions of copper-catalyzed azide−alkyne cycloaddition (CuAAC).[32] Nine different probes **13**−**21** were obtained in 7−67% yield after purification by RP HPLC (Figure 1, Table S2). Both BODIPY FL GTPγS (**2**, 71%) and probe **4** (85%) were synthesized by S-alkylation of GTPγS with BODIPY FL iodoacetamide (**1**) and 5-(iodoacetamido)-fluorescein (**3**), respectively (Figure 1 and Table S2), based on the procedures by Draganescu et al.[50] and Trans et al.,[51] with BODIPY FL GTPγS (**2**) being used as a reference probe. All final products were characterized by RP HPLC and HRMS (Figures S11−S16 and S29−S50).

Binding of Guanine Nucleotide Analogues to Gαi1. In order to provide an accurate and detailed insight into the interaction between the Gαi1 protein and the individual fluorescent guanine nucleotides (BODIPY FL GTPγS (**2**), **4** and **13**−**21**, Figure 1), we exploited the advantages of fluorescence- and, in particular, FA-based assays (e.g., direct and quantitative evaluation of the binding equilibrium, insensitive to photobleaching).[22] All measurements were performed at excitation and emission wavelengths of 485 and 528 nm, respectively, that is, close to the respective maximum of the BODIPY FL and FAM moieties of the probes (Figure S2). Preliminary dissociation constants (K_D) of the FAM-containing probes for three preparations of Gαi1 (lots 1−3) were determined and compared with those of the reference probe **2**[9] by measuring total fluorescence intensities and FA. For probes **2**, **4**, **15**, **16**, **18**, **20**, and **21**, we observed an increase in fluorescence intensity upon binding to the protein (ratio of fluorescence intensities of protein-bound and free probe $Q_b/Q_f > 1$, quantum yield enhancement factor[27]), which required correction of measured values of FA by using the ratio Q_b/Q_f for the calculation of K_D (Figure S3a,c,i,k,o,s,u). Preliminary experiments with probe **17** did not reveal such change in fluorescence intensity due to very low absolute values of total fluorescence intensity in combination with a low signal-to-noise ratio at a probe concentration (LT) of 5 nM (Figure S3m), which is why such corrective measures were not applied at this point. The low probe concentration of 5 nM in comparison to those of **2**, **4**, **15**, and **16** (50−100 nM) was, however, required to precisely determine the affinity of **17** and also those of **18**−**21**.[20,52] Even though depletion of Gαi1 due to binding to the fluorescent probe (resulting in $P_{free} < P_{total}$ at LT $\gtrsim K_D$) was considered when calculating K_D according to eqs S5−S7, with lowering the concentration of the probe having no effect on the value of the dissociation constant (data not shown), LT should not exceed ∼10 × K_D to avoid "quasi-stoichiometric titration conditions".[20,52] To ensure that the binding of the probes to Gαi1 was at equilibrium, K_D values were determined directly after mixing the two components (0 min) as well as after 33, 66, 99, and 132 min of pre-incubation at 30 °C. For the majority of the probes, relatively constant values of pK_D were obtained after 66 min (Figure S4), which is in line with the pre-incubation period of 60 min reported by McEwen et al.[9] for **2** on Gαi1. In accordance with previous reports,[46,53] the high binding affinity of the guanine nucleotide probe to Gαi1 required the presence of a low concentration of Mg^{2+} (1 mM); increasing the concentration of Mg^{2+} to 10 mM did not further improve probe affinity (data not shown). We, therefore, kept the concentration of Mg^{2+} at 1 mM in all experiments. Measurement of total fluorescence intensity of **2** (50 nM) after 66 min of pre-incubation in the presence of increasing amounts of Gαi1 revealed a K_D of 251 ± 78 nM, which is largely in agreement with the earlier reported dissociation constant of 150 ± 50 nM[9] obtained by varying the probe concentration at a constant amount of Gαi1.

Analysis of the corrected FA values gave a similar K_D value for probe **2** (228 ± 72 nM). However, closer inspection of the respective binding curves revealed a decrease in signal of the fully bound probe over time that occurred to a much lesser extent for values of total fluorescence intensity (Figures S3b vs S3a). This behavior of both total fluorescence intensity and FA was confirmed when following the respective signal over a period of 300 min at a concentration of 2.64 μM of active Gαi1, where almost all of **2** is bound to the protein (Table S4, Figure S5). As reported by Jameson et al.,[48] BODIPY FL GTPγS is slowly hydrolyzed by large amounts of Gαi1 to GDP and BODIPY FL thiophosphate, with the fluorescence of the latter product being similar to that of Gαi1-bound BODIPY FL GTPγS. Therefore, probe hydrolysis is hardly detectable by measuring total fluorescence intensities but would lead to a decrease of FA over time due to the release of low-molecular weight BODIPY FL thiophosphate from the protein. While our observations support this hypothesis, with the FA of BODIPY FL GTPγS (**2**) being calculated to drop to a level (47 mA) close to that of the unbound probe (38 ± 3 mA), a study by Tõntson et al.[19] suggested that the first-order decline of FA of BODIPY FL GTPγS (**2**) upon incubation with Gαi1 to be caused by "thermal inactivation" of the protein. Indeed, our data also correspond to a first-order decline of FA of **2** (k_{obs} = 5.23 × 10^{-3} min^{-1}), but the decrease in FA over time is much less distinct for the majority of GTP-derived probes (k_{obs} = 0.655−1.70 × 10^{-3} min^{-1}) with the exception of **4** (k_{obs} = 52.1 × 10^{-3} min^{-1}) and **15** (k_{obs} = 8.68 × 10^{-3} min^{-1}) (Table S4, Figure S5b). Consequently, this result is rather in line with the hydrolysis of the probe than with a release of the intact probe

https://doi.org/10.1021/acs.analchem.2c03176
Anal. Chem. 2022, 94, 14410−14418

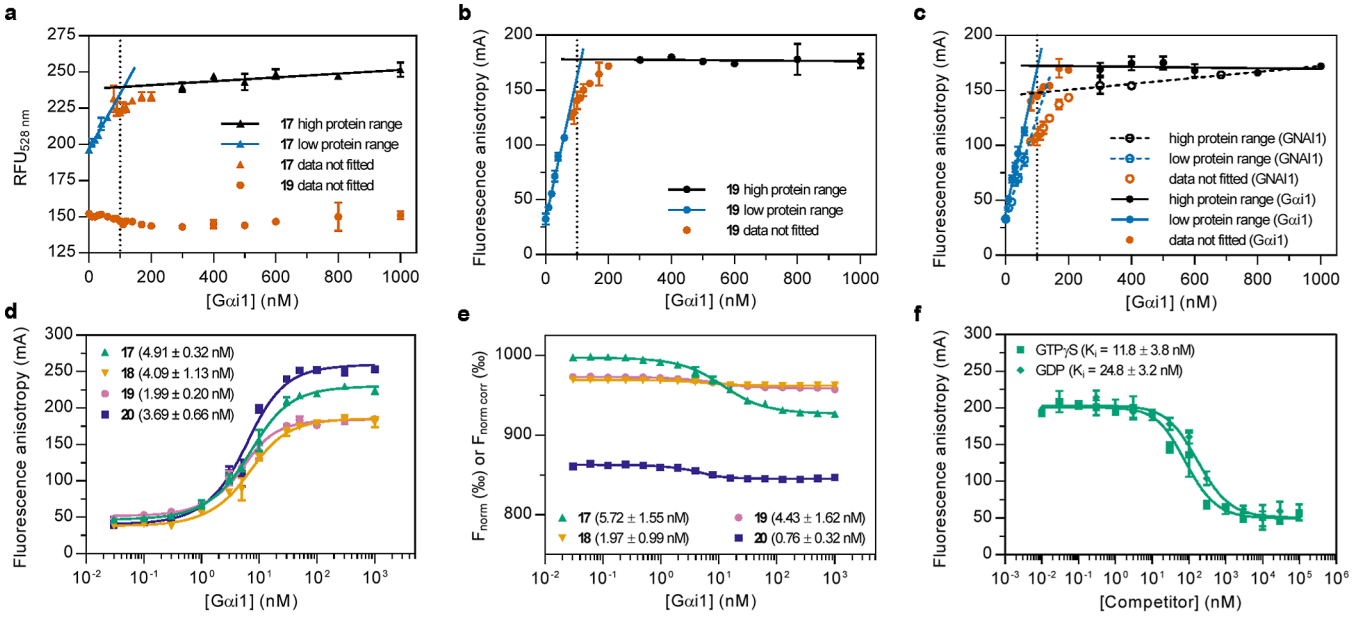

Figure 2. Binding studies and functional analysis of Gαi1 with probes **17**–**20**. (a–c) Determination of active fraction of in-house-prepared Gαi1 and commercially obtained recombinant human GNAI1 in the absence (a,b) and presence of 1% (v/v) glycerol (c) using total fluorescence intensities of 100 nM **17** (a) and values of FA of 100 nM **19** (b,c) upon addition of Gαi1. Data shown are mean values ± SD of one exemplary duplicate experiment and were obtained after 66 min of pre-incubation. Fraction of active protein was calculated from the probe concentration divided by the *x*-value of the intersection point of the two lines, with the dotted line representing the theoretical *x*-value obtained with 100% active protein.[20] (d,e) FA (d) and MST/TRIC (e) experiments to determine the dissociation constants, K_D (mean values ± SEM, n = 3–4), for the binding of the probes **17**–**20** (5 nM) to Gαi1. (d) Data shown are mean values ± SEM of three to four duplicate experiments performed after 66 min of pre-incubation. (e) The hot regions of 0.5 to 1.5 s (**17**–**19**) or 19–20 s (**20**) after switching on the IR laser were analyzed; data shown are mean values ± SEM of three experiments performed after 70 min of pre-incubation. (f) Displacement of probe **17** (5 nM) from Gαi1 (20 nM) by GTPγS or GDP. Data shown are mean values ± SEM of three duplicate experiments performed after 66 min of pre-incubation.

due to decomposition of the protein, as the latter event should similarly affect the FA of all probes over time. Furthermore, the decrease in the FA of both **2** and **15** is most prominent at concentrations of Gαi1 where the probes are completely protein-bound (Figure S3b,j), while total fluorescence intensities at low protein concentrations do not change over time (Figure S3a,i). This rather points to pronounced enzyme-mediated probe hydrolysis than to simple non-enzymatic decay. Our conclusion is supported by HPLC experiments with probes **17** and **19** showing small but progressive degradation in the presence of Gαi1, which is significantly larger than non-enzymatic probe hydrolysis (Figure S6). In contrast to these results, probe **4**, that is, the 5-amino-fluorescein analogue of **2**, undergoes both extensive non-enzymatic hydrolysis (as shown by the strong increase of total fluorescence intensity over time in the absence and presence of low concentrations of Gαi1, Figures S3c and S5a) and rapid Gαi1-catalyzed cleavage (visible by a fast decrease of FA over time at high protein concentration, Figures S3d and S5b). Because of the low stability of **4**, we were not able to thoroughly characterize the probe's binding affinity to Gαi1 in dependence on incubation time (Table S3).

To identify fluorescein probes with increased affinity and hydrolytic stability, we investigated FAM-containing analogues of GDP (**13**, **14**) and GTP (**15**–**21**) (Table S3, Figure S3). The binding of the probes to Gαi1 is mostly dependent on the presence of a γ-phosphoryl group, as the two GDP analogues are completely inactive, whereas their GTP counterparts show values of K_D in the intermediate nM range (**13** vs **15**, **14** vs **16**). Based on previously reported interactions within the nucleotide pocket (Thr181 and Gly203[54]), the presence of both the γ-phosphoryl group and several flexible atoms due to an increased linker length is expected to be favorable for nucleotide binding affinity to the protein. Moreover, taking into consideration the spatial arrangement of the respective analogue moieties (Figures S7 and S8), one could hypothesize that the extended linker size, as in probes **17**–**21**, would fit into the pocket cavity with relatively high affinity, while preventing the "bulky" FAM moiety from colliding with the Gαi1. Concomitantly, it can be concluded that the orientation of the 5-FAM moiety is preferred over that of the 6-FAM derivative in terms of probe affinity. Nevertheless, future computational analyses testing the above hypotheses are needed to gain further insight into probe–protein interactions. To increase both probe affinity and stability, the total linker length between the GTP and the FAM moiety was increased either by the introduction of two methylene groups between the GTP and the triazole ring (**15** vs **16**) or by extending the linker between the GTP-attached triazole and the FAM moiety from two to four methylene groups (**15** vs **17**), with the latter strategy being the more effective one (Tables S3 and S4). Further increase of linker length between the triazole and the FAM moiety by another two methylene groups (**16** vs **18**; **17** vs **19**, **21**) does not affect probe stability to a great extent. The same applies to the binding affinity of probes with a GTP-attached triazole, with 5-FAM (**19**) appearing to be superior in comparison to 6-FAM (**21**). In contrast, we observed an increase in binding affinity by almost an order of magnitude for **18** (vs **16**), with probes **18** and **19** being comparable in both total linker length (Figure S8) and K_D value. Additional linker elongation in **18** yielded probe **20**, which exhibits similar affinity and stability to **18** (Tables S3 and S4). Increased probe

https://doi.org/10.1021/acs.analchem.2c03176
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affinity required reduction of probe concentration for precise affinity measurements,[52] which led to a decrease of total fluorescence signal. As the FA was hereby not affected due to its ratiometric character,[55] we chose this readout for further characterization of protein−probe interaction. Probes **17**−**20** showed the most favorable properties, that is, considerably increased stability and affinity toward Gαi1 (single-digit nM K_D values) in comparison to **2** (Tables S3 and S4). Therefore, these four probes were selected for further experiments on Gαi1, exploring their binding behavior in more detail, analyzing their suitability for characterization of competitors, and determining the fraction of active Gαi1 capable of binding of GTP and probes derived thereof.

To exactly calculate the dissociation constant (K_D) of **17**−**20** on Gαi1, it was necessary to first determine the fraction of active protein (FR). This parameter represents the portion of a protein capable of interacting with ligands and is therefore crucial for all experiments and calculations where the exact concentration of binding-competent Gαi1 is required.[20] Owing to limitations concerning the detection of the GTP-binding activity of expressed Gα proteins, such as non-specific binding,[8] very little is known about the usefulness of the quantitative analysis of the guanine nucleotide binding by sophisticated methods. At present, there are only approximation methods available to determine Gαi proteins' guanine nucleotide binding activity, such as the aforementioned intrinsic tryptophan fluorescence assay[56] or radioactive[8,57,58] GTPγS binding. However, these approaches are characterized by inaccuracies due to low fluorescence intensities and small changes in fluorescence signals from tryptophan(s) and can be affected by background ionizing radiation, respectively.[8,11] To accurately quantify the fraction of active Gαi1 (lots 2 and 3) by spectrophotometric methods, we applied probes **17** and **19** at a concentration of 100 nM, which is ∼20−50 times larger than the respective K_D value, allowing for "quasi-stoichiometric titration" of Gαi1[20,52] (Figure 2a−c). When recording the total fluorescence intensity of **17** and **19** upon binding to Gαi1 (Figure 2a), we observed an increase in the total fluorescence intensity of fully protein-bound **17** (mean values ± SEM: lot 2, Q_b/Q_f = 1.32 ± 0.03, n = 6; lot 3, Q_b/Q_f = 1.30 ± 0.01, n = 3) that was not detected in previous experiments at a probe concentration of 5 nM (see above), whereas the quantum yield of **19** was not affected by the addition of protein (Q_b/Q_f = 1). This difference in Q_b/Q_f of **17** and **19** might result from the extended linker between the guanosine nucleotide and the dye for the latter probe, which probably reduces the interaction of the dye moiety with the protein. This hypothesis is supported by probe **18**, which is comparable to **19** in total linker length (Figure S8) and shows a ratio Q_b/Q_f of 1.099 ± 0.004, n = 4 (Figure S3o). However, further elongation of the linker, as in **20**, leads to an increase of Q_b/Q_f to 1.202 ± 0.002, n = 4 (Figure S3s), probably due to backfolding of the dye moiety and interaction with the protein surface. Out of the probes **17**−**20**, **17** is the most suitable for fluorescence-based experiments due to the highest ratio of Q_b/Q_f, whereas **19** is most ideal for FA assays as a correction of measured values of FA is not required.

For the determination of FR, two distinct ranges of Gαi1 were defined in the plots shown in Figure 2a−c: (i) a lower range (0−60 nM), where the concentration of Gαi1 is smaller than the probe concentration and thus added (active) protein becomes completely bound to the probe leading to a linear increase in readout signal, and (ii) a higher range (≥300 nM of Gαi1), where the concentration of Gαi1 is larger than the probe concentration and thus all of the probes is protein-bound, with adding further protein having no effect on the readout.[20] Analysis by linear regression gave two concurrent lines, whose point of intersection (x-value) represents the total protein concentration where every molecule of the probe is bound by one molecule of active Gαi1 (assuming 1:1 binding). The fraction of active protein was then calculated as the ratio of the total probe concentration and x-value. For lot 2 and lot 3 of Gαi1, we determined fractions of active protein (mean values ± SEM) of 0.69 ± 0.05, n = 6, and 0.87 ± 0.06, n = 3, respectively, using total fluorescence intensity of probe **17** (Figure 2a). The same methodology was applied to the FA of probe **19** (Figure 2b), resulting in almost identical values for the fraction of active Gαi1 (0.75 ± 0.05, n = 2, and 0.88 ± 0.05, n = 4).

Probe **19** was then applied to compare the active fraction of lot 3 of Gαi1 to that of a commercial Gαi1 protein (recombinant human GNAI1) expressed under similar conditions (Figure 2c). Experiments were performed in the presence of 1% (v/v) glycerol, originating from the commercial protein's storage buffer, yielding fractions of active protein of 1.00 and 0.75, respectively (Table S5). This result confirms that our bacterial expression protocol provides Gαi1 with a high portion of the active protein that even exceeds that of Gαi1 from a commercial source by 33%. The small increase in the active fraction of lot 3 of Gαi1 due to the addition of 1% (v/v) glycerol was attributed to the known protein-stabilizing effect of this co-solvent.[59−62] It should, however, be noted that the fraction of active Gαi1 also increased when extending the pre-incubation period to 99 and 132 min, with values even exceeding 1.00 (Table S5). This observation supports our hypothesis that longer pre-incubation periods lead to probe degradation rather than protein decomposition (Figure S5), as the latter event should decrease the fraction of active protein. In contrast, probe decay reduces the amount of protein necessary for complete binding of the probe and thus leads to an overestimation of the active fraction when including the uncorrected probe concentration into the calculation. All further experiments with probes **17**−**20** were therefore conducted after 66 or 70 min of pre-incubation time.

Knowing the fraction of active protein as well as the ratio Q_b/Q_f of the probes, we determined the corrected K_D values of **17**−**20** on lots 2 and 3 of Gαi1. FA measurements (Figure 2d) provided dissociation constants of 1.99−4.91 nM, which were similar to previously obtained preliminary K_D values (Table S3). The results of the FA assay were confirmed by MST/TRIC[63] experiments (Figure 2e, Table S6), yielding dissociation constants of 0.76−5.72 nM, respectively, with further information being provided in the Supporting Information (Figure S9). Probes **17**−**20** were identified as high-affinity ligands for Gαi1, which are superior to both BODIPY FL-[9] and MANT-labeled[16] guanine nucleotides by one to three orders of magnitude. Although these four probes showed increased stability compared to BODIPY FL GTPγS (**2**), slow degradation was observed at equilibrium with Gαi1 (beginning at about 70 min after mixing probe and protein, Table S6, Figure S5b), resulting in a limitation of the incubation period to 66−70 min to obtain reliable results. Next, we investigated the utilization of the FA assay for identifying competitors at the GTP binding site by displacement of **17** from Gαi1 by the known ligands GTPγS and GDP (Figure 2f). The dissociation constants K_i calculated for GTPγS (12 nM) and GDP (25

https://doi.org/10.1021/acs.analchem.2c03176
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nM)[64,65] are in good agreement with previously reported values on Gαi1 determined by assays following binding or enzymatic hydrolysis of radiolabeled ligands (GTPγS: 10 nM,[13] 12 nM;[46] GDP: 25 nM[46]). These data suggest that the FA-based displacement assay is highly suitable for future identification and characterization of ligands at the GTP binding site of Gαi1.

GTPase Activity of Gαi1. According to the results discussed above, we hypothesized that Gαi1 is able to hydrolyze the investigated fluorescent probes. The commercially available GTPase-Glo assay kit (Promega) was therefore utilized to quantify the GTPase activity of Gαi1. Here, GTP (with or without previous incubation with GTPase) is first converted into ATP upon addition of ADP by a nucleoside diphosphate kinase;[66] then, ATP is quantified by a luciferin/luciferase-containing detection reagent, resulting in a distinct luminescence signal.[15] In the present study, the manufacturer's protocol was modified based on Veloria et al.[67,68] by adding the 20-fold amount of ADP to guarantee complete conversion of (residual) GTP into ATP and thus increase the range of linear interdependency between the amount of GTP and the luminescence signal (Figure S10a,b). The intrinsic GTPase activity [in the absence of both GTPase activating protein (GAP) and guanine nucleotide exchange factor (GEF)] was investigated by analyzing the decrease of the luminescence signal due to the incubation of GTP with increasing amounts of lot 3 of active Gαi1 for 60 min (in the absence of glycerol), resulting in an EC_{50} of 765 ng (Figure S10c) that is similar to the reported value of 602 ng.[15] The same methodology was applied to recombinant human wild-type Ras (in the presence of 25% (v/v) glycerol originating from the commercial protein's storage buffer), which also confirms data from the literature (Figure S10d).[15] From these experiments, we calculated the amounts of remaining (protein-unbound) GTP and plotted them versus the respective total amount of GTPase per well (Figure S10e,f). The data obtained for amounts of GTP down to 20 pmol show linear behavior to the amount of protein (insets in Figure S10e,f), providing negative slopes with absolute values of 0.83 and 1.03 pmol GTP consumed over a period of 60 min per pmol of Gαi1 (lot 3) and Ras, respectively. The former value is well in agreement with the results from our experiments to determine the active fraction of lot 3 of Gαi1 with probes **17** and **19** after 66 min of pre-incubation time in the absence of glycerol (values of FR of 0.87 and 0.88, respectively) (Figure 2a,b and Table S5). Applying an FR of 0.88 to the concentration of Gαi1 in Figure S10e resulted in Figure 3 and provided a negative slope with the absolute value of 0.95 ± 0.14 pmol GTP $pmol^{-1}$ lot 3 of active Gαi1, which is not significantly different from one, meaning that every molecule of active Gαi1 is able to bind one molecule of GTP. The specific activities of lot 3 of active Gαi1 and Ras calculated from the absolute values of the slopes in Figures 3 and S10f are 0.016 and 0.017 min^{-1}, respectively. Lot 2 of active Gαi1 (in the absence of glycerol) exhibits a similar specific activity as lot 3 of 0.018 min^{-1} which is, however, reduced by about half (to 0.0085 min^{-1}) upon the addition of 25% of glycerol to the reaction mixture. The herein determined specific activities for Gαi1 (in the absence of glycerol) confirm previous data by Zielinski et al.[69] (0.016 min^{-1}), who determined the GTPase activity by following the generation of GDP. The specific activity obtained for Ras is also well in agreement with reported results in the absence of glycerol from Temeles et al.[70] (0.0177 min^{-1}) and Manne et al.[71] (~1.2 pmol GTP mol^{-1} GTPase per 60 min), indicating that 25% of glycerol has no effect on the GTPase activity of Ras. It should be noted that steady-state measurements following multiple turnover GTPase reactions of Gαi1 (including GDP/GTP exchange and GTP hydrolysis) were shown to be rate-limited by the nucleotide exchange, in particular by the release of GDP.[58,72,73] Therefore, turnover numbers from such steady-state experiments mostly reflect the rate constants of GDP release.[58,72,74] Our experiments with both Gαi1 and Ras revealed that further increasing the protein amount beyond 35 pmol leads to a deviation from the linear decrease in GTP, with even 140−150 pmol of GTPase not being able to completely consume the substrate (vermillion data in Figures 3 and S10e,f). The observed behavior at high protein concentrations can be explained by a lowered enzymatic turnover rate due to substrate depletion and/or the formation of "higher order oligomeric species".[75]

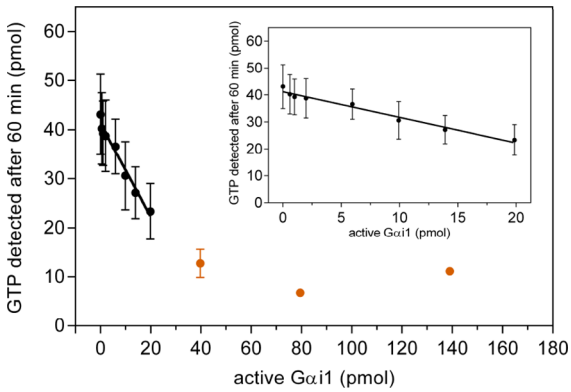

Figure 3. GTPase activity of Gαi1. Amounts of GTP (mean values ± SEM, $n = 3$) detected after 60 min pre-incubation of GTP (50 pmol) with increasing amounts of active Gαi1 were calculated from the luminescence intensities in Figure S10c and the linear equation in Figure S10a. Linear regression of data (black) in the range of 0−19.9 pmol lot 3 of Gαi1 (magnified in the inset) gave 0.95 ± 0.14 pmol GTP $pmol^{-1}$ Gαi1 (mean value ± SEM, $n = 3$) as an absolute value of the negative slope. For lot 2, the corresponding absolute values of the slopes in the absence and presence of 25% of glycerol were 1.09 ± 0.07 and 0.51 ± 0.07 pmol GTP $pmol^{-1}$ Gαi1 (mean values ± SEM, $n = 2−3$), respectively (data not shown). Vermillion data were not used for data analysis.

CONCLUSIONS

In our study, we aimed to establish an efficient bacterial expression protocol for highly active Gαi1 together with a robust method allowing for the determination of the fraction of Gα protein with GTP binding activity. To the best of our knowledge, we herein introduce the first non-radioactive approach for Gαi1 to determine the fraction of active protein, which was driven by the discovery of the high-affinity probes **17** and **19**. The determination can be reproducibly performed in low volume (20 μL) by a one-step homogeneous assay on a 384-well plate and is, therefore, less laborious and time-consuming than radiometric assays. The introduced FA assay, in combination with the optimized expression and purification of Gαi1, offers the opportunity to study the modulation of Gαi1 by ligands and other protein partners in a setup that provides comparable results to radioligand binding assays but does not include the challenges of working with radioactivity. Starting from the results reported herein, we consider the development of efficient strategies to produce active and

https://doi.org/10.1021/acs.analchem.2c03176
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soluble Gα proteins from other subfamilies, such as Gαs and Gα12/13, in combination with the discovery of respective ligands by applying the FA assay as future milestones to understand the regulation of G protein signaling cascades.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c03176.

Standard experimental details and additional data supporting this article, complete data sets of protein production and characterization (CD, MALDI, and SDS-PAGE), nucleotide analogue synthesis (NMR, RP-HPLC profiles, and high-resolution mass spectra), binding curves, probe stability studies, computational analyses, MST/TRIC studies, and GTPase activity assays (PDF)

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#A.P., P.S., and R.K. contributed equally. D.I., M.P., and J.J. conceived the research and designed the experimental studies with the support of A.P., P.S., R.K., and J.K.; A.P. developed and performed all required experiments for Gαi1 protein production and characterization with the support of T.K. A.P., P.S., and M.P. performed and analyzed the fluorescence spectroscopy, FA, MST/TRIC measurements as well as the GTPase-Glo assays; F.A.B. performed computational analyses. C.H., R.L., R.W., P.S., and M.P. developed the FA assay. R.K. and M.C. synthesized the guanine nucleotide derivatives. M.B. performed HPLC experiments for investigating probe stability and analyzed the data; A.P. and P.S. collected the data of the functional and binding assays and analyzed the data with D.I., M.P., and J.J. All authors discussed the results, contributed to and have given approval to the final manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support of the University of Bonn, the Deutsche Forschungsgemeinschaft (DFG) within FOR 2372 and IM 97/14-1 (to D.I.), the Faculty of Medicine and University Hospital Cologne (to M.P.), the University of Warsaw (to J.J.), and the National Science Centre (UMO-2015/18/E/ST5/00555 to J.K.) is gratefully acknowledged. CD and MST/TRIC measurements were performed at the Protein Interaction Platform Cologne (http://PIPC.uni-koeln.de). Technical assistance by S. Linden and B. Nubbemeyer (University of Bonn) is also gratefully acknowledged.

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https://doi.org/10.1021/acs.analchem.2c03176
Anal. Chem. 2022, 94, 14410−14418

Appendix C: In-depth Characterization of Gαs Protein Activity by Probing Different Guanine Nucleotides

A. Pepanian, P. Sommerfeld, F. A. Binbay, D. Fischer, M. Pietsch, and D. Imhof. In-depth characterization of Gαs protein activity by probing different guanine nucleotides. (**2023**).

This manuscript was submitted to *Protein Science* on July 20, 2023.

In-depth characterization of Gαs protein activity by probing different guanine nucleotides

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Running title: Gαs protein characterization with nucleotide probes

Total number of manuscript pages: 37

Number of figures and tables in main text: 5 figures

Total number of supplementary material pages: 18

Number of figures and tables in the supplementary material: 9 figures, 1 table

Abstract:

The heterotrimeric G proteins are found in all eukaryotic cells as key interacting partners of G protein-coupled receptors (GPCRs). Upon G protein activation, the ability

of the Gα subunit to exchange GDP for GTP determines the intracellular signal transduction. Although various studies have successfully shown that both Gαs and Gαi have an opposite effect on the intracellular cAMP production, with the latter being commonly described as “more active”, the functional analysis of Gαs is a comparably more complicated matter. Additionally, the thorough investigation of the ubiquitously expressed splice forms of Gαs, Gαs(short) and Gαs(long), is still pending. Since the previous experimental evaluation of the activity and function of the Gαs isoforms is not consistent, the focus was laid on structural investigations to understand the GTPase activity. Herein, we examined recombinant human Gαs by applying a recently established methodological setup developed for Gαi characterization. The ability for GTP binding was evaluated with fluorescence and fluorescence anisotropy assays, whereas the intrinsic hydrolytic activity of the two isoforms was determined by a GTPase assay. Among different nucleotide probes, including MANT-GTPγS and two known Gαi1 binders, BODIPY FL GTPγS exhibited the highest binding affinity towards the Gαs subunit. Overall, this work delivers a deeper understanding of the Gαs subunit, also providing novel information concerning the differences between the two protein variants.

Keywords: BODIPY FL GTPγS, Fluorescence Anisotropy, G proteins, Gαs(long), Gαs(short), GTPase-Glo assays, MANT-GTPγS, MD simulations

Statement of importance: The production of high amounts of the soluble and active Gαs(long) and (short) is not yet successful. Assays denoted the controversies regarding the functional behavior of both isoforms indicating a flexible α-helical domain. Herein, an efficient protein production as well as bioanalytical, biochemical, and functional characterization methodology via fluorescence anisotropy is described.

The functional differences between the two homologs are evaluated by binding affinities to guanine nucleotide analogs.

Abbreviations: AC, adenylyl cyclase; AHD, α-helical domain; α-H: α-helices; β_2-AR, β_2-adrenergic receptor CD, circular dichroism; cAMP, cyclic AMP; DHAP, 2,5-dihydroxy-acetophenone; *E. coli*, *Escherichia coli*; FA, fluorescence anisotropy; Gαs(l), Gαs(long); Gαs(l) Refld., Gαs(long) refolded; Gαs(s), Gαs(short); GPCR, G protein-coupled receptor, G proteins, guanine nucleotide-binding proteins; 6xHis-tag, hexahistidine tag; IMAC, immobilized metal affinity chromatography; K_D, equilibrium dissociation constant; MD, molecular dynamics; R.c., random coil; SEC, size exclusion chromatography; SI, supplementary information; SW, switch regions.

Introduction

Ligand binding to the seven transmembrane G protein-coupled receptors (GPCRs) stimulates the activation of the heterotrimeric guanine nucleotide-binding proteins (G proteins) leading to their dissociation into the Gα·GDP (inactive state) and the Gβγ dimer[1]. Therefore, the nature of the guanosine nucleotide (GDP or GTP) is a natural determinant of the function of the Gα proteins and, subsequently, of the stimulated intracellular signaling cascade[2,3]. GTP binding to the Gαs protein subunit (one of the four main subfamilies of the Gα proteins)[4] activates adenylyl cyclase (AC), leading to the subsequent increase in concentration of the second messenger, cAMP (cyclic adenosine monophosphate)[5]. On the contrary, the Gαi functions by inhibiting the formation of cAMP in the cells[6]. For many decades, scientists in several fields devoted themselves to assessing and modulating the Gα proteins' functional activity[2,7–11].

We recently reported a methodology for enhancing the total yield of the recombinant human Gαi1 protein subunit and evaluated its activity by quantifying the active protein fraction using fluorescence and fluorescence anisotropy (FA) assays[11]. An abundant amount of existing research asserts the more challenging expression and general production of Gαs compared to Gαi1, with a particular focus on bacterial expression systems[12–18] and some reports indicating that attaining a soluble protein fraction is not feasible due to the formation of inclusion bodies[18,19]. The "susceptibility" of the Gαs protein for aggregation in *Escherichia coli* (*E. coli*) cells derives from the flexibility of the α-helical domain (AHD)[20,21], as well as from natural variants[22]. Therefore, it is no surprise that specific expression conditions (low temperature and low IPTG concentration[12,14–16] as well as gentle centrifugation[23]) are required to maintain the protein's integrity. Proper folding is essential for the protein to fulfill its biological duties. Attempts to increase Gαs activity via protein refolding were somewhat abortive[18]. The

indisputable low yields of recombinant Gαs limited its further characterization for several years[12,18,19]. This accounts for the numerous studies in which the Gαs activity was assessed or analyzed in the presence of the Gβγ dimer[113,14], the β_2-adrenoreceptor (β_2-AR)[20,24–27] or generally in the membrane context (upon protein modification[28]). Consequently, less frequent are the cases where a human, unmodified (native) Gαs protein has been recombinantly produced in bacteria[15, 19,20,29,30].

Apart from the above reports, the heterogeneity of the protein has been described since the early 1980's[13,14,31,32]. Two Gαs isoforms (short and long), which result from mRNA splicing[33] in exon 3, are ubiquitously expressed in almost all tissues[34]. Although minor differences between the two isoforms have been reported, including, e.g., the faster kinetics of GDP dissociation for the Gαs(long) version[13,14,34], the eminence of the short variant for biochemical and structural studies is evident[18, 20, 28,29,35–40]. This can be exemplified by the crystal structures available for Gαs(short)·GDP (PDB:6EG8[27]) and Gαs(short)·GTPγS (PDB:1AZT[41]), whereas for the Gαs(long) protein, only an AlphaFold structure (Uniprot ID: P63092, corresponding to P63092-1) is available. However, a careful review of the literature reveals that there is an actual controversy concerning the functional evaluation of the two isoforms[34], since the Gαs(short):Gαs(long) ratio was found to be associated with elevated[42], stable[43] or diminished[44] AC activity or intrinsic enzyme activity[34]. The additional 14-15mer sequence in the case of Gαs(long) is in close proximity to the linker I region (located between the AHD and the GTPase-domain). Yet, an explicit explanation of the functional and structural relevance of the two homologs has been pending, if not paused, for the last two decades.

Although the Gαi1 subunit exhibits a higher binding affinity towards guanine nucleotides compared to Gαs[14,45–47], it is compelling to explore the structural

determinants deciphering the functional differences. For instance, it is known that a specific segment of the AHD (residues 65-150) is not conserved in most Gα subunits, with Gαs being the most diverse among all[48]. The α3-β5 and α4-β6 loops are the main different regions between Gαs and Gαi, which accounts for the distinct receptor and effector binding ability[41,49]. In parallel, two motifs (NKXD and TCAX[50]) are crucial for GDP binding in the nucleotide pocket. However, a common denominator is that the vast majority of both experimental and structural studies are conducted in a receptor (β_2-AR)- or effector (AC)-dependent manner in membrane or cellular studies. We recently established an efficient protocol for the recombinant expression of the human Gαi1 protein in a bacterial system resulting in high yield and quality of the protein[11]. Gαi1 was characterized in detail by fluorescence intensity-, FA-, and luminescence intensity-based methods. As a result, the nucleotides **17** and **19** (introduced in Pepanian *et al*.[11]) showed K_D values in the low nM range, which were appox. two orders of magnitude lower than that for the well-established BODIPY FL GTPγS. The high affinity allowed for the determination of the active fraction (~69-88%) of the Gαi1 protein. Concerning the activity evaluation of Gαs, initial trials with radioactive GTPγS isotopes[12–16,18] or with indirect assays (such as trypsin digestion[18]), and later with fluorescently-labeled GTP analogs (such as MANT GTPγS or BODIPY FL GTPγS)[22, 26,36] have been performed, lacking though the determination of the actual protein activity. Therefore, we applied the methodological setup[11] to characterize and accurately quantify the nucleotide binding to recombinantly expressed human Gαs(short) and Gαs(long) variants and their GTPase activities.

Results

Expression, purification, and characterization of Gαs protein isoforms.

With the intention to thoroughly characterize the Gαs protein function *in vitro*, we performed the expression of the human His_6-tagged Gαs(short and long) proteins without any modifications, such as myristoylation, in *E. coli* cells[25,28]. The production protocols were inspired by former reports with minor modifications[12, 20, 36,51]. For induction of protein expression, a variety of IPTG concentrations (30 µM, 100 µM, 250 µM, and 500 µM, Fig. S1) was used, with the highest yield being obtained with 30 µM IPTG (30 ˚C for 9 h) which was then kept consistent for the expression of both isoforms. The soluble protein samples were subjected to a two-step purification protocol (IMAC and SEC), with the final protein samples eluted with 250 mM imidazole and 50 µM GDP[11,20]. The individual fractions of the SEC profile (Fig. 1a) were analyzed, combined, and concentrated. We observed that a fraction of the protein was accumulated in the pellet in the form of inclusion bodies and thus decided to recover it by employing a refolding protocol (modified from refs.[17,18,52]). Therefore, the protein was first solubilized with 6 M guanidinium chloride (Gdn-HCl) and then refolded via stepwise dialysis (6 to 0 M Gdn-HCl). However, the protein tended to rapidly aggregate after ultra-centrifugation, indicating incorrect protein folding despite the refolding attempt. The purity of all final protein samples (native and refolded), their molar masses, and the N-terminal sequences were validated by SDS-PAGE (Fig. 1b), MALDI-TOF-MS (Fig. 1c, d), and Edman sequencing, respectively. The theoretical molar mass of the Gαs(short) and Gαs(long) variants is 48341.49 g mol^{-1} and 49739.96 g mol^{-1}, respectively. The protein production resulted in 2.5-2.9 mg L^{-1} of native Gαs(long)·GDP, 5.0–6.1 mg L^{-1} refolded Gαs(long)·GDP, and 4.0–4.8 mg L^{-1} native Gαs(short)·GDP. Noteworthy, the struggle of obtaining adequate yields of the

Gαs proteins compared to Gαi1 (a similar approach gave a ca. 10-fold higher yield for Gαi1[11, 18,53]) still remained the limiting factor throughout the study. Due to the tendency of the Gαs proteins to precipitate with time, CD analysis was conducted with the native proteins Gαs(long)·GDP and Gαs(short)·GDP as well as the refolded Gαs(long)·GDP to investigate the secondary structure and overall protein conformation, as reported earlier (Fig. 1e)[17].

In addition, and to the best of our knowledge, no data has been published regarding the conformation of Gαs(long)·GDP (for both, the native and the refolded, states) that could be used for comparison. Therefore, we performed computational studies using the structures or structural models available so far (Fig. 2)[27,54]. According to the structural alignment, the secondary structural elements of Gα subunits are conserved. However, deviations between the structures occur due to the flexible loop regions such as linker I, the switch regions (SW) and the α4-β6 loop. Interestingly, the linker I of the long isoform (14 residues longer) showed that some residues (Pro77-Ser84) expand the existing α-helix (αA of AHD, Fig. 2a, c). As a result, our analysis (resulting in 31% α-helices and 18% β-sheets)[17] for Gαs(short)·GDP (Fig. 1f) are in good agreement with the previously reported data (far-UV data: 36% α-helices and 18% β-sheets)[17]. Although we expected a higher α-helical character for the long isoform (due to the elongated sequence in the AHD), the protein conformation seems to adopt a more random structure with a higher β-sheet character, which could be supported by the computational studies where the additional residues of the long variant were conformationally changed within AHD. The CD results of the refolded Gαs(long)·GDP indicated a radical loss of conformational rigidity, which, in combination with the laborious production, prevented us to proceed further with the refolded Gαs(long) protein. Concerning functional analysis, we thus focused on native Gαs(short) and

Gαs(long) only. A sequence alignment of both Gαs homologs with Gαi1 can be found in Fig. S2.

Binding of guanine nucleotide analogs to Gαs protein isoforms.

We intended to directly compare the binding behavior of distinct nucleotide probes towards the Gαs variants with that of Gαi1 using the experimental setup described earlier[11]. Therefore, fluorescence and FA-based assays were applied to both Gαs(short) and (long). Initially, we tested the widely used[18, 21,22, 26, 28, 36,55], non-radioactive probe BODIPY FL GTPγS. In contrast to Gαi1, efficient nucleotide binding required an increased concentration of $MgCl_2$ (10 mM instead of 1 mM[11]), as was also reported by others[13, 15, 20, 28, 30, 40,56,57]. Bokoch *et al.*[45] already mentioned the higher binding affinity of the Gαi protein for nucleotide analogs compared to Gαs, which is also in agreement with other studies[14,47]. Only McEwen *et al*.[28] demonstrated a stronger binding of BODIPY FL GTPγS to Gαs(short) compared to Gαi (70 vs. 150 nM), with both proteins being myristoylated, which could affect their functional behavior. To the best of our knowledge, no other dissociation constant for binding of BODIPY FL GTPγS to Gαs has been reported so far, which triggered us to investigate not only qualitatively but also quantitatively the binding affinity to this nucleotide derivative. Besides, Gαs is prone to bind guanine nucleotides in a fast manner[21], with Gαs(long) exhibiting faster kinetics compared to the short isoform[14]. Therefore, we followed the time-dependent change in FA (Fig. 3a, d) and fluorescence (Fig. 3b, e) for binding of BODIPY FL GTPγS to either the long or the short Gαs isoform. As found for Gαi1[11], ligand binding was fast, with signals peaking after ~120 s (long) and ~240 s (short) (Fig. 3a, b, d, e), respectively as similarly observed in the past[21,22]. At the signal peak, values of both FA and fluorescence increased with added Gαs protein, following a saturation function as shown in Fig. 3c, f.

Interestingly, the FA decreased towards the value of unbound probe according to first-order kinetics after the signal peak had been reached (Fig. 3a, d), with first-order rate constants (Fig. S3a, c) showing a tendency to decrease with increasing protein concentrations. This observation can be interpreted as time-dependent dissociation of a fluorescent species from Gαs; and it does not support the hypothesis of a protein-catalyzed probe-decay releasing the fluorescence moiety from the protein as the rate of such enzymatic cleavage should increase with the Gαs concentration.

The total fluorescence intensity also dropped after ~120 s (Gαs(long)) and ~240 s (Gαs(short)), respectively, but did not reach the plateau of the non-enzymatic control (Fig. 3b, e).

A similar behavior of FA and total fluorescence intensity was observed for the probes MANT-GTPγS (Fig. S4a), **17**, and **19** (Fig. S5a, b). MANT-GTPγS is considered to be a stable ligand of Gα proteins with regards to hydrolysis of the βγ-diphosphoester bond[25,58,59]. The results obtained for all the probes investigated herein do not favor Gαs-mediated probe-decay, but rather indicate a less stable protein-probe complex, compared to Gαi. Interaction of the probes with the two Gαs isoforms was quantified by calculation of the dissociation constant (K_D) at the time of maximum probe binding using the values of the total fluorescence intensity. BODIPY FL GTPγS was the most potent probe, exhibiting stronger affinity on Gαs(short) than on Gαs(long) (1.98 vs. 5.66 μM) (Fig. 3c, f) as previously also shown by Gille *et al*.[25] for both Gαs isoforms fused to membrane-bound β_2-AR. The obtained K_D values were confirmed by FA measurements performed in the same experiments (1.62 vs. 6.92 μM, Fig. S3b, d). MANT-GTPγS and the two high-affinity Gαi1 probes **17** and **19**[11] were weaker binders for Gαs(short) with K_D values of 6.17 μM (Fig. S4b), 18.4 μM, and 7.22 μM (Fig. S5c), respectively.

Gαs(short) and BODIPY FL GTPγS were selected to study the direct effect of the known competitor GTPγS on probe-protein interaction. Increasing concentrations of GTPγS prevented binding of the probe to the protein, shown as decrease in the signal maximum of fluorescence and FA, respectively, at 240 s (Fig. 3g, h) with an IC_{50} value of 0.34 µM (Fig. 3i, S3f). This inhibition constant points to a considerably higher affinity of GTPγS in comparison to BODIPY FL GTPγS and MANT-GTPγS, which is in line with previous reports on both Gαs(short) and Gαs(long)[25,26]. FA traces (Fig. 3g) in the presence of GTPγS showed a similar behavior compared to its absence, while the concentration of GTPγS did not influence the first-order rate constant of proposed probe release from protein (k_{obs}) to a great extent (Fig S3e). This observation is supported by the course of the fluorescence traces in Fig. 3h, which tend to converge at a later time as expected for experiments with identical probe and protein concentrations.

Computational characterization of nucleotide binding to Gαs isoforms.

The binding affinity of nucleotide analogs to different Gα protein subunits is known to be different[45]. However, the residues surrounding the nucleotide-binding pocket are highly conserved between the Gαi1 and Gαs(short) subunits[41] and are in contact with particular regions of the nucleotide[60]. *In silico* studies were carried out to compare the binding profiles of the nucleotides to Gαs(short) and Gαs(long). However, without available structural data for the Gαs(long) protein, a direct comparison between the isoforms is not trivial. Basic computational investigations, including molecular docking and dynamic (MD) simulations (Supporting Information text), were initially performed on both Gαs subunits (apo state) with the nucleotide analogs GTPγS and BODIPY FL GTPγS (Fig. 4a-d) to derive structural information and explanations of the results obtained from the binding studies (Fig. 3).

The MD simulation of the Gαs(short)·GTPγS complex structure confirmed the interaction of GTPγS with the well-known conserved residues[8,41]. Essential residues involved in this interaction are: Glu50, Gly52, Lys53, Ser54, and Thr55 in the P-loop region, Leu198 and Arg199 in the αF of AHD, and Asn292, Lys293, and Asp295 in the NKXD motif, all of which occur with a frequency >70% (Fig. 4a). Although MD simulation of the complex of Gαs(short) with BODIPY FL GTPγS showed that the BODIPY FL moiety participates only infrequently in hydrophobic interactions with the residues Gly49, Gly226, and Arg228, the core part of the nucleotide (GTPγS) was stabilized by multiple hydrogen bonding interactions (Gly52, Lys53, and Thr55 in the P-loop; Leu198 and Arg199 in the αF; Asn292 and Asp295 in the NKXD motif) plus a hydrophobic interaction (Lys293 in the NKXD motif) (Fig. 4b). The core of BODIPY FL GTPγS, in comparison to GTPγS, was engaged in less and slightly weaker interactions due to the flexible BODIPY FL moiety. These results are in accordance with our binding studies, where GTPγS is indeed a good competitor of the Gαs(short)-BODIPY FL GTPγS interaction. MD simulation of the Gαs(long)-BODIPY FL GTP□S complex compared to the short isoform revealed that the core part of the nucleotide is stabilized by a strong contact with Asp295, but a weaker interaction with Asn292(Fig. 4b,d). Moreover, the interactions of αF residues with the ribose were found frequently (50-60%). Although the phosphate group is stabilized by the residues Gly52, Ser54 and Thr55, the frequencies of their interactions are somewhat modest. Nevertheless, the BODIPY FL moiety is involved in stabilizing interactions with the residues Leu203 (SWI) and Arg258 (SWIII). The simulation trajectory supported the higher stability of the Gαs(short)-BODIPY FL GTPγS complex compared to the complex of the probe with Gαs(long).

Computational studies were also conducted for the probes MANT-GTPγS (Fig. S6-S7), analog **17** (Fig. S8a), and **19**[11] (Fig. S8b) binding to Gαs(short), which similarly to the binding studies revealed only an insufficient probability for binding. Stereochemical hindrance of the respective fluorophore moiety indicated unstable interactions and/or spontaneous probe dissociation. Further details concerning the individual probes are summarized in the Supporting Information. This strengthens our assumption that BODIPY FL GTPγS and GTPγS are the recommended probes to investigate both Gαs isoforms and computationally supports the experimental selectivity of compounds **17** and **19** towards the Gαi1 subunit[11].

GTPase activity of the Gαs protein isoforms.

Another significant property of the Gαs proteins is their GTPase activity, which was quantified by means of the GTPase-Glo assay (Promega) as previously reported for the Gαi1 subunit[11]. We determined the GTPase turnover of both Gαs(short) and Gαs(long) after incubation times at which the maximum binding of BODIPY FL GTPγS to the respective isoform had been observed (2 min for the long and 4 min for the short isoform). In the GTPase-Glo assay, remaining GTP is converted to ATP, which is then quantified based on the luminescence signal produced by luciferase[11,61]. No effector (e.g., receptor, accessory protein, or similar) was involved in the GTPase measurements. The intrinsic GTPase activity was evaluated by plotting the remaining amount of GTP (calculated from luminescence intensities by means of the calibration curve in Fig. S9) versus the respective total amount of Gαs protein per well and linear regression of the data (Fig. 5). The enzymatic activity was obtained as absolute value of the negative slope. A linear interdependency of GTP and protein concentration was found for 0−12.1 pmol of Gαs(long) (Fig. 5a) and 0−41.3 pmol of Gαs(short) (Fig. 5b) resulting in specific activities of 1.7 and 0.13 min^{-1}, respectively. Since we were not

able to determine the fractions of active protein for the two Gαs isoforms (as was previously possible for Gαi1[11]), the numbers of the two specific Gαs activities cannot directly be compared with each other. However, the specific activity of Gαs(short) was found to be well in agreement with that determined by Graziano *et al*.[14] (0.12 min^{-1}) and Lee *et al*.[62] (~0.15 min^{-1}). In contrast, Gαs(long) is ca. 5.5-times more active than reported before[14,29], with the specific activity larger than one pointing to multiple GTP turnover. Nevertheless, faster GTP hydrolysis as observed for the long isoform confirms previous studies[14], where the long isoform showed accelerated binding of GTPγS and a higher GTPase activity.

CONCLUSIONS

In our previous study, we characterized the functional activity of the Gαi1 subunit with two high-affinity binders **17** & **19**[11]. Herein, we applied the same methodology to unravel the so-far uncovered activity of human Gαs(short) and Gαs(long) recombinantly expressed in bacteria and free of other modifications, such as myristolyation. The present work provides an efficient protocol producing the two Gαs isoforms in high yields (compared to existing strategies) and proves distinct properties and functional differences between the short and long homologs. Both isoforms exhibited fast binding of the nucleotide probe BODIPY FL GTPγS, however, with much higher values of K_D compared to Gαi1[11]. Nevertheless, BODIPY FL GTPγS has a higher affinity towards the short isoform than MANT-GTPγS, **17** and **19**, with GTPγS being a potent probe competitor. The observed selectivity of probes **17** and **19** for the Gαi1 protein over the Gαs isoforms (>1000-fold) is impressive, implying that moiety size is somehow irrelevant for the nucleotide-binding cavity but more a matter of interactions with the neighboring residues. This observation comes in accordance with our computational studies, where GTPγS and BODIPY FL GTPγS exhibited frequent and stable interactions with several protein residues. Noteworthy, this work provides the first computational hints on BODIPY FL GTPγS interactions with both Gαs isoforms. We speculate that the flexibility of the proteins' AHD and that of the BODIPY FL GTPγS-protein complex limited previous attempts for complex crystallography which would shed light on comprehending the interactions within the binding pocket. Finally, the GTPase-Glo assay confirmed the GTPase activity of the two Gαs proteins, with that of the short isoform being minor to the activity of the long homolog. Altogether, this study offers an in-depth experimental and computational characterization as well as a comparison of Gαs(short) and Gαs(long) subunits, suggesting the fast and

preferable binding of BODIPY FL GTPγS compared to other fluorescently labeled nucleotide probes. Consequently, as the aforementioned set of assays was suitable not only for the Gαi1 but also for Gαs proteins, other Gα protein subfamilies could also be investigated in the future.

Materials and methods

Bacterial expression and purification of soluble Gαs proteins. The gene of human Gαs isoform 1 (Gαs(long), Uniprot ID: P63092-1) or human Gαs isoform 2 (Gαs(short), Uniprot ID: P63092-2) was cloned into the pET28a(+) vector[20, 36,51], containing an N-terminal (hexahistidine) 6xHis-tag and an enterokinase cleavage site (Eurofins Genomics GmbH, Germany). The plasmid encoding the desired protein was transformed into *E. coli* BL21(DE3) bacteria cells as previously described with slight modifications[12, 20, 36, 51,63]. Bacteria were first grown in LB medium (50 µg mL^{-1} kanamycin, 30 °C, 15 h) and then cultured in an "enriched" LB medium (with 2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM KH_2PO_4 pH 7.2, 50 µg mL^{-1} kanamycin, 30 °C) up to an OD_{600} of 0.6. Protein expression was induced by addition of 30 µM IPTG (30 °C, 14 h), then the cells were harvested, and the pellets stored at -80 °C. Various IPTG concentrations (30–500 µM) were tested for the protein induction, keeping, finally, 30 µM IPTG consistent throughout the further expressions. Reported protocols[12, 18,36] were considered concerning the importance of GDP and $MgCl_2$ addition in each step. Bacterial cells were lysed using 1 mg mL^{-1} of lysozyme and a protease inhibitor mixture (Aprotinin, Pefabloc, Pepstatin and phenylmethylsulfonyl fluoride (PMSF)). The expressed proteins were first purified by Ni-NTA affinity chromatography (immobilized metal affinity chromatography, IMAC) followed by size exclusion chromatography (SEC) on an Äkta Prime Plus device equipped with a HiLoadTM 16/600 Superdex column. The obtained fractions were analyzed for protein concentration (Bradford assay using Roti-Nanoquant[64]) and purity (SDS-PAGE), combined and stored at -80 °C. All protein aliquots were stored in elution buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 50 µM GDP, and 250 mM imidazole) after 10% glycerol was added. Prior to further experiments, a buffer

exchange was carried out (Amicon Ultra-15 centrifugal filter unit with 30 kDa cut-off) as described previously[11]. For the final protein centrifugation, it is recommended to spin the protein down at 4400 – 6000 rpm in 2 minutes-increments until the desired final volume is obtained, as described in the literature[23]. Details on the gene sequence and the protein refolding (when applied) are provided in the Supporting Information (SI).

Biophysical protein characterization. All Gαs protein samples were buffer exchanged into ddH_2O[11], and the respective molar mass was detected via MALDI-TOF-MS using 2,5-dihydroxy-acetophenone as the matrix. The N-terminal sequence was confirmed by Edman sequencing on a PPSQ-53A protein sequencer (Shimadzu, Duisburg, Germany). The secondary structure of the soluble and refolded proteins was estimated with circular dichroism (CD) spectroscopy using a JASCO model J-715 spectrometer as previously described[11] with 5 µM of the respective Gαs protein sample in 10 mM sodium phosphate pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, and 50 µM GDP. The data evaluation was facilitated by Dichroweb[65] and the K2D2 algorithm[66]. Details are listed in the SI.

Fluorescence- and FA-based binding assays. The synthesis and characterization of the fluorescently labeled nucleotide analogs **17** and **19** are described in Pepanian *et al*.[11]. BODIPY FL GTPγS sodium salt (5 mM in 50 mM Tris, pH 7.5) was a gift from Jacek Jemielity (Centre of New Technologies, University of Warsaw, Poland), commercially obtained from ThermoFisher Scientific. MANT-GTPγS triethylammonium salt (10 mM aqueous solution) and GTPγS were from Jena Bioscience (Jena, Germany). Details on the probes are shown in Table S1. The binding studies with Gαs(long) or Gαs(short) and the nucleotide probes BODIPY FL GTPγS (50 nM), **17** (5 nM), **19** (5 nM), and MANT-GTPγS (200 nM), respectively, were performed as

previously described with few alterations[11]. Both Gαs isoforms were freshly buffer exchanged into 20 mM HEPES pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT (exchange buffer), with 5% (v/v) DMSO and 0.05% (v/v) Tween 20 being added afterwards. All measurements were done in a total volume of 20 µL at 30 °C on a BioTek Synergy 2 multimode microplate reader equipped with the software Gen 5 version 1.11.5. in black, non-binding, 384-well round-bottom low-volume PS plates (Corning®, product no. 4514) at excitation and emission wavelengths of 485 nm and 528 nm (BODIPY FL GTPγS, **17**, **19**) or 360 nm and 460 nm (MANT-GTPγS), respectively. Competitition experiments followed binding of BODIPY FL GTPγS (50 nM) to Gαs(short) (3000 nM) in the prescence of GTPγS (10-30000 nM). Negative and positives controls included probe alone and protein and probe, respectively. The same volume (10 µL) of 2x (double-concentrated) protein and 2x probe (with or without 2x competitor) were mixed in the wells, and the total fluorescence intensity (sensitivity was as follows: BODIPY FL GTPγS, **17**, and **19**: 55; MANT-GTPγS: 85), as well as both parallel and perpendicular fluorescence intensities (for determination of FA) were measured directly (0 s) and every 60 s for 3600-18000 s. Further details on the data analyses are available in the SI.

Description of supplementary material:

Supporting information Text

Experimental studies

***In silico* characterization of the binding of nucleotide probes**

Acknowledgments

Financial support by the Deutsche Forschungsgemeinschaft (DFG) within IM 97/14-1 (to D.I.), the Faculty of Medicine of the University of Cologne, and the University Hospital Cologne (to M.P., D.F.), is gratefully acknowledged. CD measurements were performed at the Protein Interaction Platform Cologne (http://PIPC.uni-koeln.de). The authors thank J. Jemielity (Centre of New Technologies, University of Warsaw, Poland) for providing BODIPY FL GTPγS, probes **17** and **19**, as well as T. Kühl, S. Linden, and Y. Elsayed (University of Bonn) for technical assistance.

Author contributions

D.I. conceived the research and designed the experimental studies with the support of A.P., P.S., and M.P.; A.P. developed and performed all experiments for Gαs protein production and characterization with the guidance of D.I.; A.P., P. S. and M.P. planned, performed and analyzed the fluorescence intensity and FA measurements as well as the GTPase-Glo assays; F.A.B. performed and evaluated the computational analyses with the support of A.P. and D.I.; A.P. and F.A.B. wrote the manuscript. M.P., P.S., D.F., and D.I. proof-read and revised the manuscript. All authors discussed the results, contributed to and approved the final manuscript.

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Figure legends

Figure 1. Gαs protein production and characterization. (a) Size exclusion chromatography (SEC) elution profiles of Gαs(long)·GDP (left) and Gαs(short)·GDP (right), respectively. Peaks designated by the dashed circles correspond to the isolated proteins as verified by SDS-PAGE (b) and MALDI MS (c, d). L: PageRuler™ Prestained Protein Ladder (10–180 kDa). (e) Circular dichroism (CD) spectra of Gαs(s) for Gαs(short)·GDP, Gαs(l) for Gαs(long)·GDP and Gαs(l) refld. for refolded Gαs(long)·GDP. (f) Secondary structure elements calculated from CD spectra. α-H: α-helices, R.c.: random coil. Previous data from Najor *et al.*[17] are used for comparison.

Figure 2. Structural comparison of different Gα subunits. (a) Gαs(short)·GTPγS (PDB: 6EG8[27], coral) and predicted Gαs(long) (Uniprot ID: P63092_1, hosted by the EBI, grey), CαRMSD= 0.830 Å. The 14 residues, not present in the short isoform are highlighted in orange in Gαs(long). (b) Gαs(short)·GTPγS (coral) and Gαi1·GTPγS (PDB:1GIA[58], dark green), CαRMSD= 1.119 Å. Residues absent in Gαi1 compared to Gαs are labeled in cyan. (c) Gαi1·GTPγS (dark green) and predicted Gαs(long) (grey), CαRMSD= 1.249 Å. In all the structures, the linker I region, switch regions (SWI-III), α3-helix, α3-β5 loop, and α4-β6 loop are highlighted in green, red, purple, yellow, and blue, respectively. Nucleotides in the Gαs(short)·GTPγS and Gαi1·GTPγS structures are illustrated in ball-and-stick style in magenta. Structural and sequence differences in linker I between the Gα-subunits are indicated in the magnified sections of (a)-(c). The whole sequence alignment can be found in Fig. S2**.**

Figure 3. Binding studies on Gαs(long) (a-c) and Gαs(short) (d-i). Time-dependent FA and fluorescence (mean value ± SEM, n = 1-3) of BODIPY FL GTPγS (50 nM) in

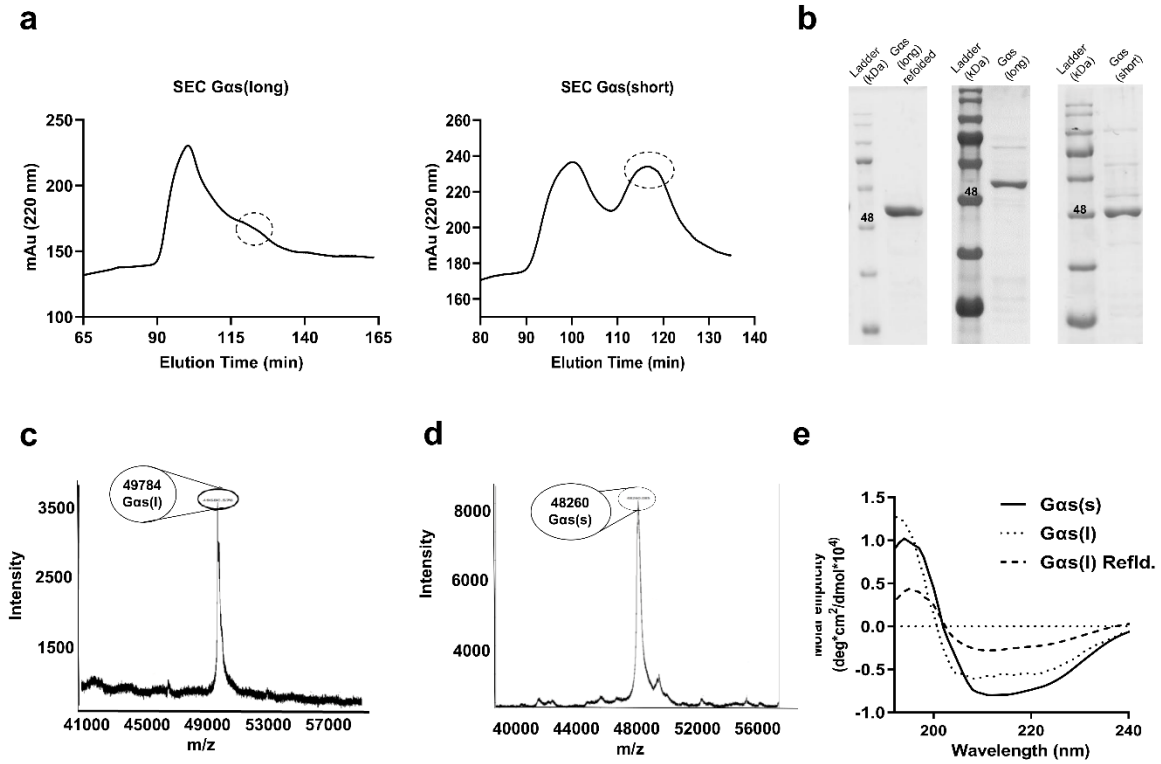
the presence of increasing concentrations of Gαs(long) (a, b) and Gαs(short) (d, e), respectively. Time-dependent FA (g) and fluorescence (h) (mean value ± SEM, n = 3) of BODIPY FL GTPγS (50 nM) for binding to Gαs(short) (3000 nM) in the presence of increasing concentrations of GTPγS. Data of FA in (a, d, g) were subjected to nonlinear regression according to a model of first-0rder decay (a: 120-3600 s; d, g: 240-5400 s). Determination of the K_D value (mean values ± SEM, n = 3) for binding of BODIPY FL GTPγS to the long (c) and short (f) isoforms. Data were taken from (b) (at 120 s, see dotted line) and (e) (at 240 s, see dotted line), respectively. (i) Determination of IC50 and n_H (mean value ± SEM, n = 3) for competition of GTPγS with BODIPY FL GTPγS for binding to Gαs(short). Data (mean values ± SEM, n = 3) were taken from (h) at 240 s (see dotted line). n_H = -1.21 ± 0.04.

Figure 4. Stable interactions of GTPγS and BODIPY FL GTPγS in the nucleotide-binding pocket of the Gαs(short) (PDB: 6EG8[27]) and the predicted Gαs(long) (UniProt ID: P63092_1) structure. Residues in the P-loop, αF, and NKXD motif with which the probe frequently interacts are depicted in cyan, red and blue, respectively. Hydrogen bonding and hydrophobic interactions between the nucleotide and the surrounding protein residues are displayed as dashed yellow and green lines, respectively. Mg^{2+} is shown as a yellow sphere. Structural (left column) and atomic (right column) representation of GTPγS or BODIPY FL GTPγS bound to the nucleotide-binding pocket of Gαs proteins are depicted. The interactions of (a) GTPγS with Gαs(short), (b) BODIPY FL GTPγS with Gαs(short), (c) GTPγS with Gαs(long) and (d) BODIPY FL GTPγS with Gαs(long) were considered.

Figure 5. **GTPase activity of Gαs isoforms.** Amounts of GTP (mean value ± SEM, n = 3) detected after 2 min (a, Gαs(long)) or 4 min (b, Gαs(short)) of pre-incubation of GTP (50 pmol) with increasing amounts of the respective Gαs isoform (total protein) were calculated from the luminescence intensities and the calibration curve in Fig. S9. Linear regression of data (black, vermillion) in the range of 0−12.1 pmol of Gαs(long) (magnified in the inset) and 0−41.3 pmol of Gαs(short) gave 3.39 ± 0.18 (black) and 3.35 ± 0.09 (vermillon) pmol GTP $pmol^{-1}$ Gαs(long) and 0.50 ± 0.04 pmol GTP $pmol^{-1}$ Gαs(short) (mean value ± SEM, n = 3) as an absolute value of the negative slope. Open circle data (black, vermillion) were not used for data analysis.

Figures

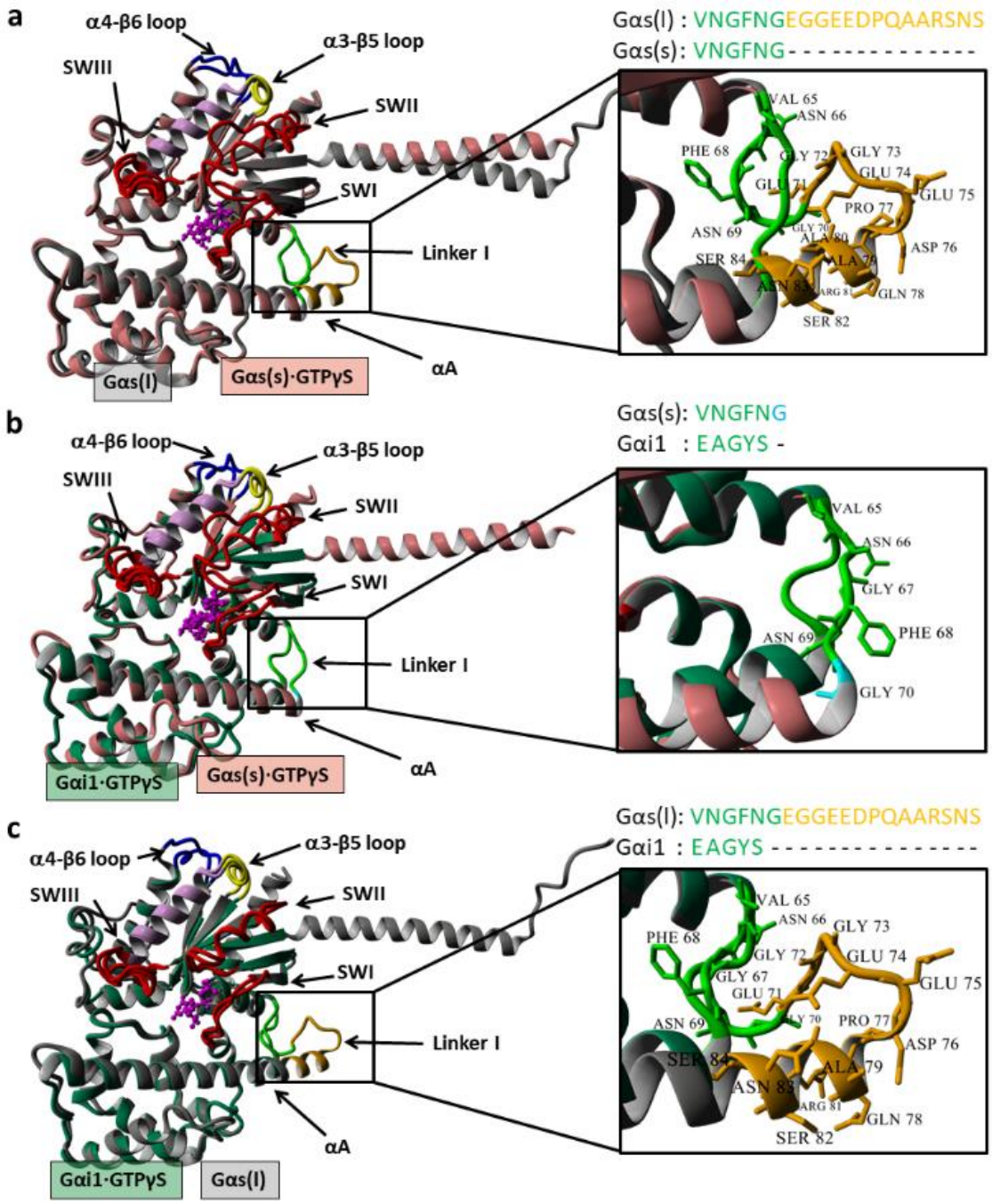
Figure 1



f

	Gαs(s) ([17])	Gαs(s)	Gαs(l)	Gαs(l) Refld.
α-H	**36%**	**31%**	**21%**	**2.64%**
β-sheet	**18%**	**18%**	**25%**	**51.8%**
R.c.	**33%**	**41%**	**64%**	**54%**

Figure 2


a
α4-β6 loop
α3-β5 loop
SWIII
SWII
SWI
Linker I
αA
Gαs(l)
Gαs(s)·GTPγS
Gαs(l) : VNGFNGEGGEEDPQAARSNS
Gαs(s): VNGFNG- - - - - - - - - - - - -
VAL 65
ASN 66
PHE 68
GLY 72
GLY 73
GLU 74
GLU 71
GLU 75
PRO 77
ASN 69
ASP 76
SER 84
GLN 78
SER 82
b
α4-β6 loop
α3-β5 loop
SWIII
SWII
SWI
Linker I
αA
Gαi1·GTPγS
Gαs(s)·GTPγS
Gαs(s): VNGFNG
Gαi1 : EAGYS -
VAL 65
ASN 66
GLY 67
ASN 69
PHE 68
GLY 70
c
α4-β6 loop
α3-β5 loop
SWIII
SWII
SWI
Linker I
αA
Gαi1·GTPγS
Gαs(l)
Gαs(l): VNGFNGEGGEEDPQAARSNS
Gαi1 : EAGYS - - - - - - - - - - - - - -
VAL 65
ASN 66
GLY 73
PHE 68
GLY 72
GLU 74
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ASP 76
SER 84
ALA 79
ASN 83
GLN 78
SER 82

Figure 3

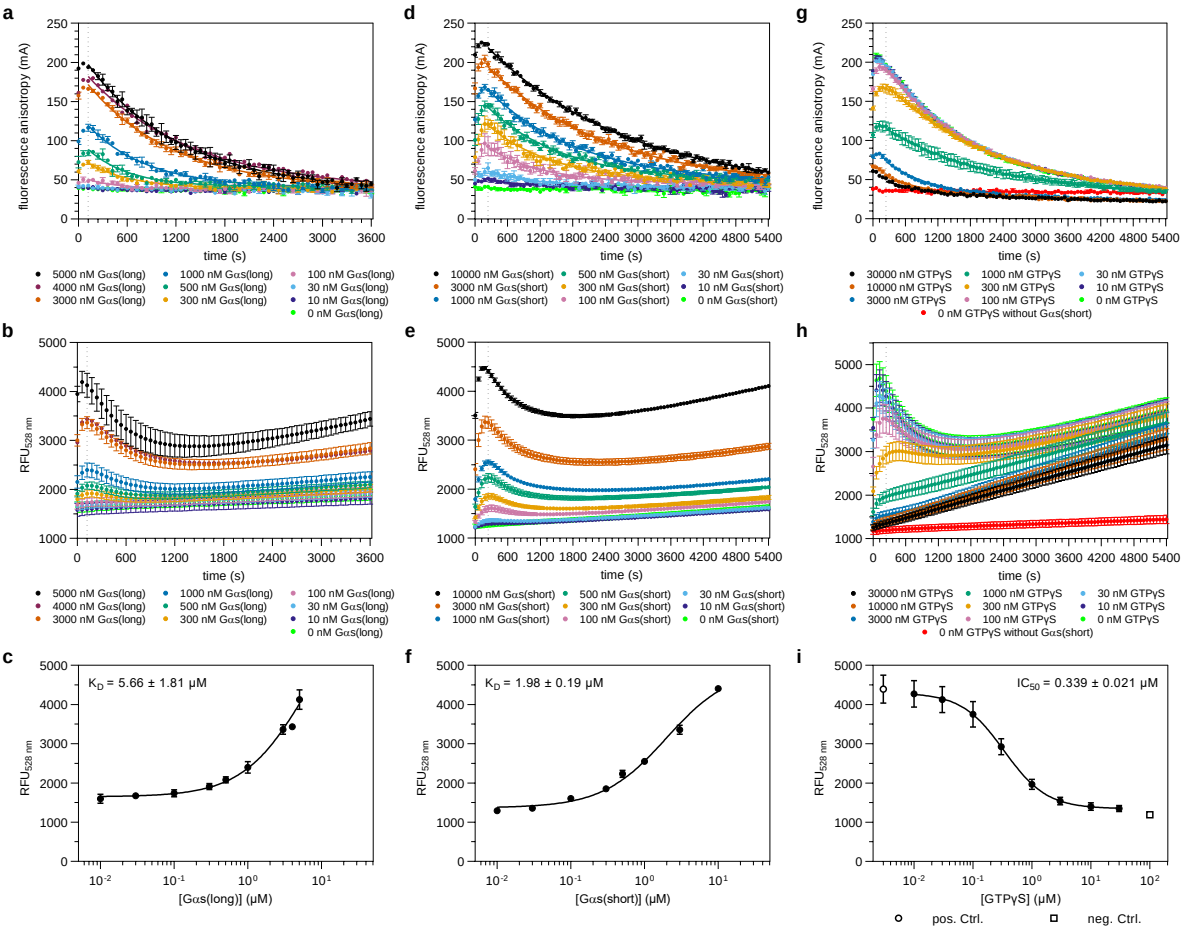
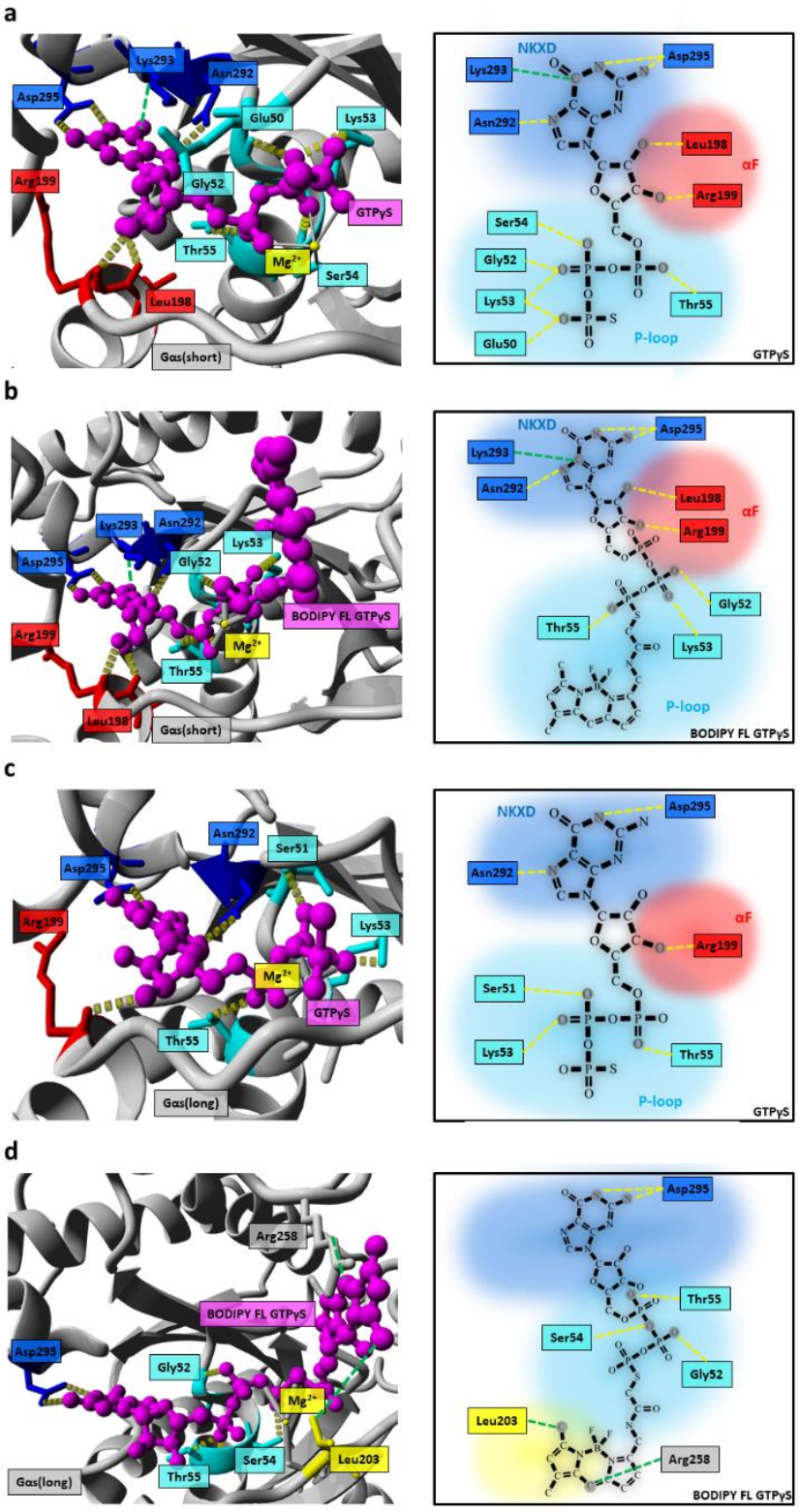

a
fluorescence anisotropy (mA)
time (s)
5000 nM Gαs(long)
4000 nM Gαs(long)
3000 nM Gαs(long)
1000 nM Gαs(long)
500 nM Gαs(long)
300 nM Gαs(long)
100 nM Gαs(long)
30 nM Gαs(long)
10 nM Gαs(long)
0 nM Gαs(long)
b
RFU528 nm
time (s)
c
KD = 5.66 ± 1.81 µM
RFU528 nm
[Gαs(long)] (µM)
d
fluorescence anisotropy (mA)
time (s)
10000 nM Gαs(short)
3000 nM Gαs(short)
1000 nM Gαs(short)
500 nM Gαs(short)
300 nM Gαs(short)
100 nM Gαs(short)
30 nM Gαs(short)
10 nM Gαs(short)
0 nM Gαs(short)
e
RFU528 nm
time (s)
f
KD = 1.98 ± 0.19 µM
RFU528 nm
[Gαs(short)] (µM)
g
fluorescence anisotropy (mA)
time (s)
30000 nM GTPγS
10000 nM GTPγS
3000 nM GTPγS
1000 nM GTPγS
300 nM GTPγS
100 nM GTPγS
30 nM GTPγS
10 nM GTPγS
0 nM GTPγS
0 nM GTPγS without Gαs(short)
h
RFU528 nm
time (s)
i
IC50 = 0.339 ± 0.021 µM
RFU528 nm
[GTPγS] (µM)
pos. Ctrl.
neg. Ctrl.

Figure 4


a
Lys293
Asn292
Asp295
Glu50
Lys53
Arg199
Gly52
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Thr55
Mg2+
Ser54
Leu198
Gαs(short)
NKXD
Asp295
Lys293
Asn292
Leu198
αF
Arg199
Ser54
Gly52
Lys53
Thr55
Glu50
P-loop
GTPγS
b
Lys293
Asn292
Lys53
Asp295
Gly52
BODIPY FL GTPγS
Arg199
Mg2+
Thr55
Leu198
Gαs(short)
NKXD
Asp295
Lys293
Asn292
Leu198
αF
Arg199
Gly52
Thr55
Lys53
P-loop
BODIPY FL GTPγS
c
Asn292
Ser51
Asp295
Arg199
Lys53
Mg2+
GTPγS
Thr55
Gαs(long)
NKXD
Asp295
Asn292
αF
Arg199
Ser51
Lys53
Thr55
P-loop
GTPγS
d
Arg258
BODIPY FL GTPγS
Asp295
Gly52
Mg2+
Ser54
Leu203
Thr55
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Thr55
Ser54
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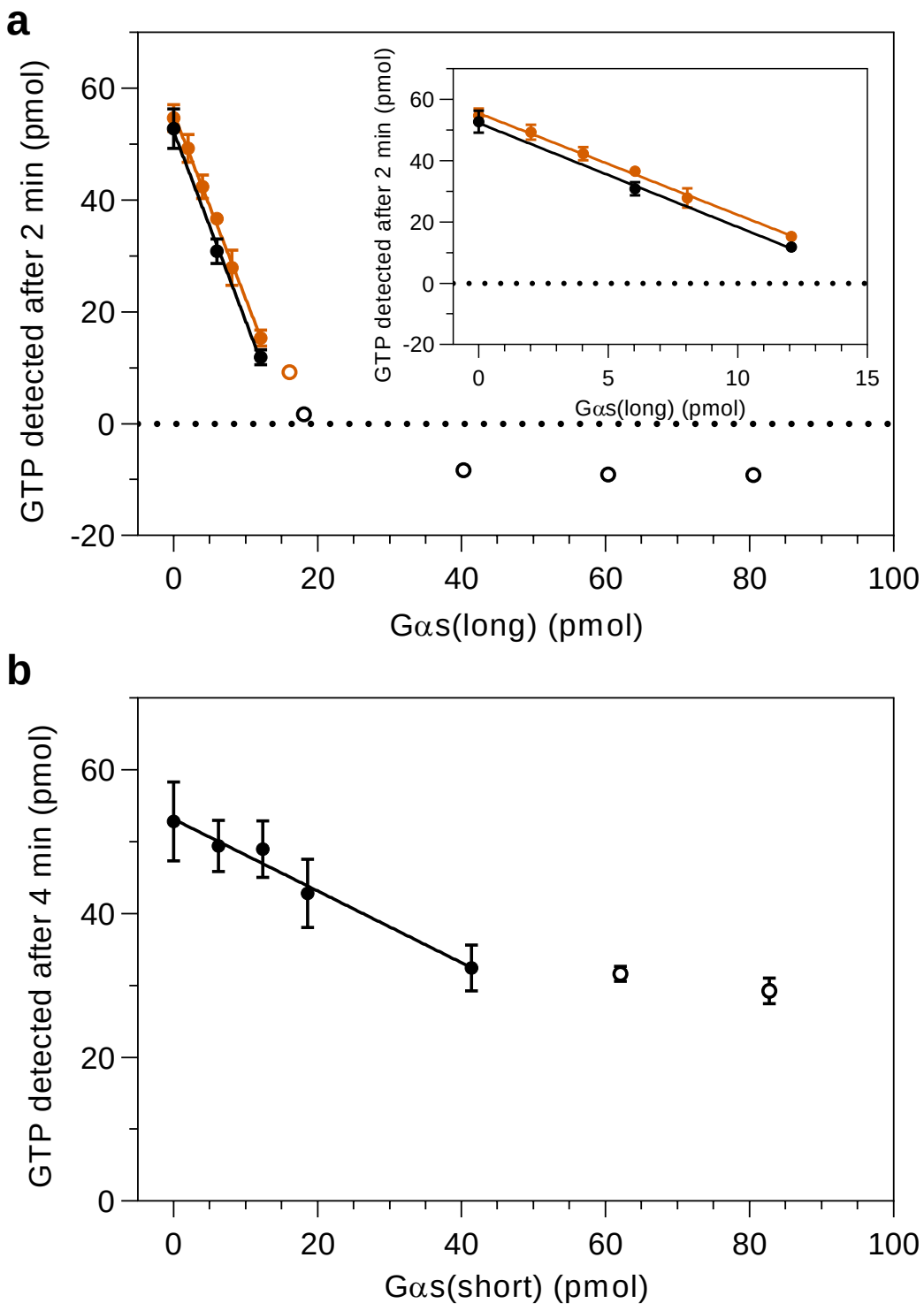
Leu203
Arg258
BODIPY FL GTPγS

Figure 5


a
GTP detected after 2 min (pmol)
60
40
20
0
-20
0
20
40
60
80
100
Gαs(long) (pmol)
GTP detected after 2 min (pmol)
60
40
20
0
-20
0
5
10
15
Gαs(long) (pmol)
b
GTP detected after 4 min (pmol)
60
40
20
0
0
20
40
60
80
100
Gαs(short) (pmol)

Appendix D: Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators

B. Nubbemeyer, A. A. Paul George, T. Kühl, A. Pepanian, M. S. Beck, R. Maghraby, M. Shetab Boushehri, M. Muehlhaupt, E. M. Pfeil, S. K. Annala, H. Ammer, D. Imhof, and D. Pei. 5. Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators. *ACS Chem. Biol.* (**2022**), 17(2), 463–473, doi: 10.1021/acschembio.1c00929.

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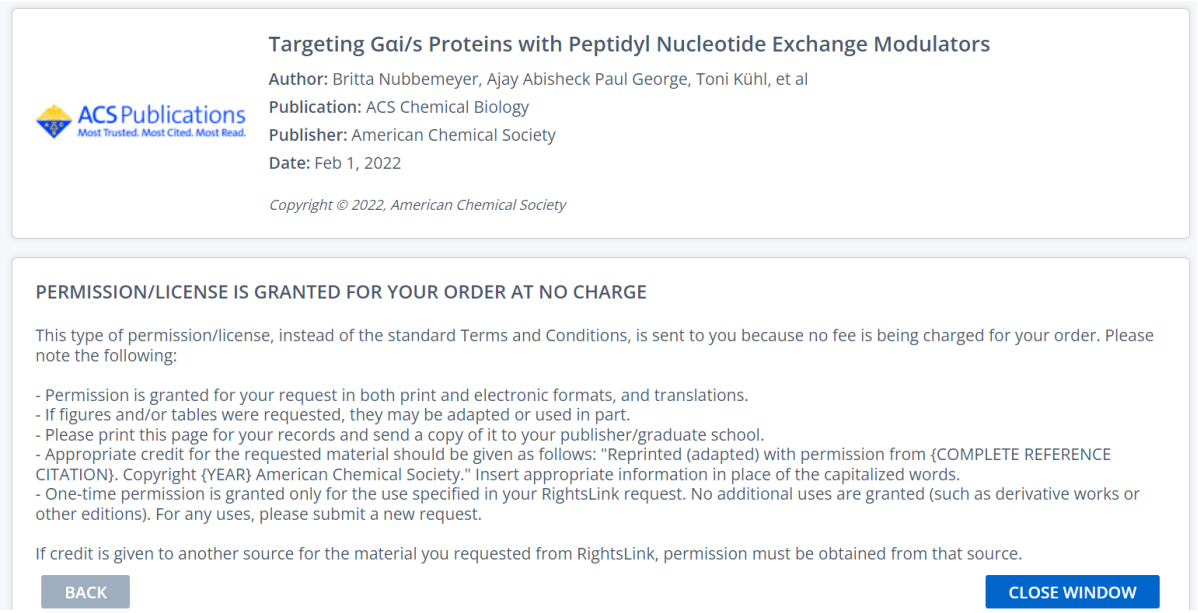

Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators

Author: Britta Nubbemeyer, Ajay Abisheck Paul George, Toni Kühl, et al

Publication: ACS Chemical Biology

Publisher: American Chemical Society

Date: Feb 1, 2022

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Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators

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Cite This: ACS Chem. Biol. 2022, 17, 463−473

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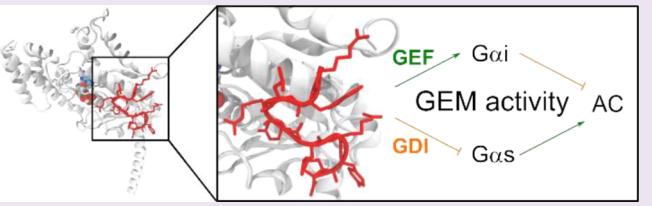
ABSTRACT: Chemical probes that specifically modulate the activity of heterotrimeric G proteins provide excellent tools for investigating G protein-mediated cell signaling. Herein, we report a family of selective peptidyl Gαi/s modulators derived from peptide library screening and optimization. Conjugation to a cell-penetrating peptide rendered the peptides cell-permeable and biologically active in cell-based assays. The peptides exhibit potent guanine-nucleotide exchange modulator-like activity toward Gαi and Gαs. Molecular docking and dynamic simulations revealed the molecular basis of the protein−ligand interactions and their effects on GDP binding. This study demonstrates the feasibility of developing direct Gαi/s modulators and provides a novel chemical probe for investigating cell signaling through GPCRs/G proteins.

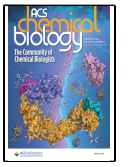


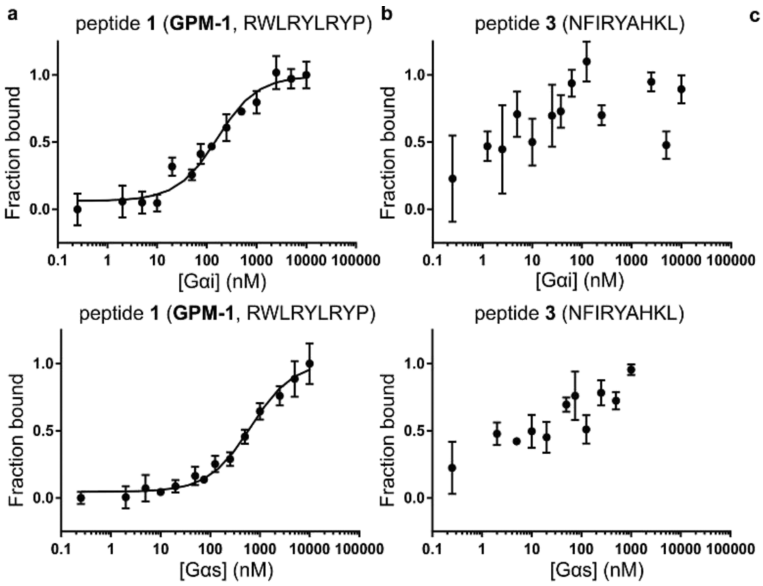
INTRODUCTION

G protein-coupled receptors (GPCRs) transmit signals into the cell via membrane-associated heterotrimeric G proteins (Gαβγ), which are classified into the four subfamilies: Gαi/o, Gαs, Gαq/11, and Gα12/13.[1] G proteins function as molecular switches by alternating between inactive guanine-nucleotide diphosphate (GDP)—bound and active guanine-nucleotide triphosphate (GTP)—bound states and interacting with different downstream effectors, e.g., adenylyl cyclase (AC). Generally, Gαs stimulates, whereas Gαi inhibits, the activity of AC, thereby modulating the intracellular cyclic adenosine monophosphate (cAMP) level.[2,3] Malfunction of GPCRs leads to a multitude of human diseases, including cancer; consequently, GPCRs have been a fertile ground for drug discovery.[4,5] Approximately 30% of FDA-approved drugs target more than 100 GPCRs.[4] However, targeting GPCRs has its limitations, as some diseases involve the malfunctioning of several GPCRs, each of which mediates a specific G protein signaling,[2,3,6] while in other cases, G proteins may initiate signaling pathways independent of GPCRs.[2,3,5−7] Directly targeting intracellular G proteins thus represents an attractive alternative to GPCR-directed therapeutics, but remains a major challenge for several reasons. First, G proteins do not have well-defined pockets on their surfaces and are difficult to target with conventional small molecules. Second, different G protein subfamilies share a high degree of sequence and structure similarity, making it difficult to specifically target a given G protein.[2,3,6] Finally, G proteins reside inside the cytosol necessitating cell permeability of any G protein modulator. To date, only a few specific G protein modulators are known, including FR900359 (FR) and YM-254890 (YM), both of which are natural products and target Gαq.[2,5−9] No such specific and selective small- or medium-sized modulators exist for targeting the intracellular Gαi and Gαs subfamilies, although protein modulators are known, e.g., cholera toxin (CTX, which is a Gαs activator) and pertussis toxin (PTX, which is a Gαi inhibitor).[3,10,11] Mastoparan and suramin compounds are notable outliers, which broad application is limited due to their lack of specificity[12,13] and selectivity,[2] respectively.

Inspired by the fact that both FR900359 (FR) and YM-254890 (YM) are cyclic peptides, researchers have begun to explore linear and cyclic peptides as potential G protein modulators, often by screening combinatorial peptide libraries. For example, screening of mRNA display libraries has identified linear peptides R6A,[14] R6A-1,[14,15] AR6-05,[16] and GSP,[17] as well as more proteolytically stable macrocyclic peptides cycGiBP,[18] cycPRP-1,[19] cycPRP-3[19] and Gα SUPR[20] as high-affinity binders for Gαi1·GDP (Figure 1d).[3,14−20] A GDP-selective ligand, KB-752,[21] was obtained from a phage display library and acts as guanine-nucleotide exchange factor (GEF) for Gαi1 and as guanine-nucleotide dissociation inhibitor (GDI) for Gαs.[21−23] This bifunctional activity is

Received: November 24, 2021
Accepted: January 6, 2022
Published: January 18, 2022





c

peptide	sequence	K_d(Gαi) [μM]	K_d(Gαs) [μM]
1	RWLRYLRYP	0.14±0.02	0.63±0.09
2	NWGAYLDGL	0.23±0.06	n.b.
3	NFIRYAHKL	n.b.	n.b.
4	WPKNYRQKI	n.b.	n.b.
5	WVQRHPYAV	n.b.	n.b.
6	FHQWHRTQK	n.b.	n.b.
7	FQYQCRKHF	0.18±0.07	0.47±0.12
8	TSRYHMHRI	n.b.	n.b.
9	MYYYHKVRH	n.b.	n.b.
10	SSMFHIGLN	0.23±0.07	n.b.
11	KVRMYEEIE	n.b.	n.b.
12	QILKYYNFT	n.b.	n.b.
13	PDRDHHKYR	n.b.	n.b.

d

										Φ	ζ	W	Φ	[+/-]	Ω	Φ							
KB-752	-	-	-	-	-	-	-	S	R	V	T	W	Y	D	F	L	M	E	D	T	K	S	R
GSP	M	A	M	S	D	R	N	K	R	L	T	V	W	E	F	L	A	L	P	S	S	T	-
R6A-1	-	-	-	-	-	-	-	D	Q	L	Y	W	W	E	Y	L	-	-	-	-	-	-	-
AR6-05	-	D	E	S	D	P	E	E	L	M	Y	W	W	E	F	L	S	E	D	P	S	S	-
cycGiBP	-	-	-	-	-	-	-	-	M	I	T	W	Y	E	F	V	A	G	T	K	-	-	-
cycPRP-1	-	-	-	-	-	-	-	-	M	I	T	W	I	D	F	I	S	P	S	K	-	-	-
cycPRP-3	-	-	-	-	-	-	-	-	-	M	T	W	F	E	F	L	S	S	T	S	K	-	-
Gα SUPR	-	-	-	-	-	-	-	-	M	F	Y	A*	Y	E	Y	A*	Q	W	S	K	-	-	-
peptide **1** (**GPM-1**)	-	-	-	-	-	-	-	-	-	-	R	W	L	R	Y	L	R	Y	P	-	-	-	-
peptide **2**	-	-	-	-	-	-	-	-	-	-	N	W	G	A	Y	L	D	G	L	-	-	-	-
peptide **7**	-	-	-	-	-	-	-	-	-	-	-	-	F	Q	Y	Q	C	R	K	H	F	-	-
peptide **10**	-	-	-	-	-	-	-	S	S	M	F	H	I	G	L	N	-	-	-	-	-	-	-

Figure 1. MST results and sequence alignment of the best Gαi1·GDP binders. (a) Binding curves of 5(6)-carboxyfluorescein-labeled peptide **1** (**GPM-1**) with Gαi1/s, error bars represent standard deviation (SD) for $n = 3$; (b) peptide **3** as nonbinding representative for Gαi1/s, $n = 2$; (c) MST results of all hits. (n.b.: no binding (<1 μM or not saturated); M = Nle). (d) Sequence alignment of KB-752,[21] GSP,[17] R6A-1,[14] AR6-05,[16] cycGiBP,[18] cycPRP-1/3,[19] Gα SUPR,[20] and peptides **1** (**GPM-1**), **2**, **7**, **10**. M = Nle[29] for peptide **10**, * = *N*-methylated amino acids, **Φ**: hydrophobic (V, I, L, F, W, Y, M), **Ω**: aromatic (F, W, Y), **ζ**: uncharged hydrophilic (N, Q, S, T), [+]: basic (H, K, R) and [−]: acidic (D,E) amino acids.[24]

referred to as the guanine-nucleotide exchange modulator (GEM) activity, which was originally described for GIV and other proteins containing a GEM motif (ΦTΦX[D/E]FΦ, where Φ is a hydrophobic residue and X is any residue).[24] The GEM motif has previously been referred to as the Gα-binding and activating (GBA) motif.[25−27] Structural analyses of the Gαi1·GDP/KB-752 and Gαi3·GDP/GIV−GEM complexes revealed that both peptides bind to a region between the Switch II motif and α3 helix of Gαi.[21,28] In this study, we discovered a relatively potent modulator against Gαi1·GDP, **GPM-1**, by screening a one-bead-one-compound (OBOC) peptide library. Subsequent optimization of **GPM-1** and conjugation with a cell-penetrating peptide (CPP) improved its binding affinity, proteolytic stability, and cell permeability. Biomolecular simulations provided insights into the molecular basis of the underlying peptide−protein interactions. Our results show that the cell-permeable variants of **GPM-1** (**GPM-1c** and **GPM-1d**) modulate the Gαi/s activity in cell culture in a GEM-like manner and thus provide a novel lead for further development into highly specific and potent Gα modulators in the future.

RESULTS AND DISCUSSION

Identification of Gαi1 Binders. The lengths/sizes of GEM motifs in proteins (7aa),[23,25] FR/YM (eight building blocks),[9] and the high-affinity Gαi1·GDP ligand R6A-1 (9aa)[14,15] suggest that a peptide sequence of seven to nine residues should be sufficient for high-affinity binding to Gα proteins. We therefore screened a previously reported nonapeptide OBOC library[29] for binding to biotinylated Gαi1·GDP by following a well-established library screening protocol (Figure S1 and text in SI).[30,31] A total of 101 hit sequences (Table S1) were obtained and analyzed for recurring motifs to establish any consensus sequence(s) (Figure S1). 13 representative peptides (**1**−**13**) were selected from different consensus groups for resynthesis and binding studies (Figures 1c and S1, Tables S2, S3).

Specificity for Gα Binding of Selected Hits. The binding affinity of the selected hits for Gαi/s was determined by microscale thermophoresis (MST, Figures 1a−c and S3) employing fluorescein-labeled peptides and varying concentrations of recombinant Gαi1·GDP or Gαs·GDP (Table S4

https://doi.org/10.1021/acschembio.1c00929
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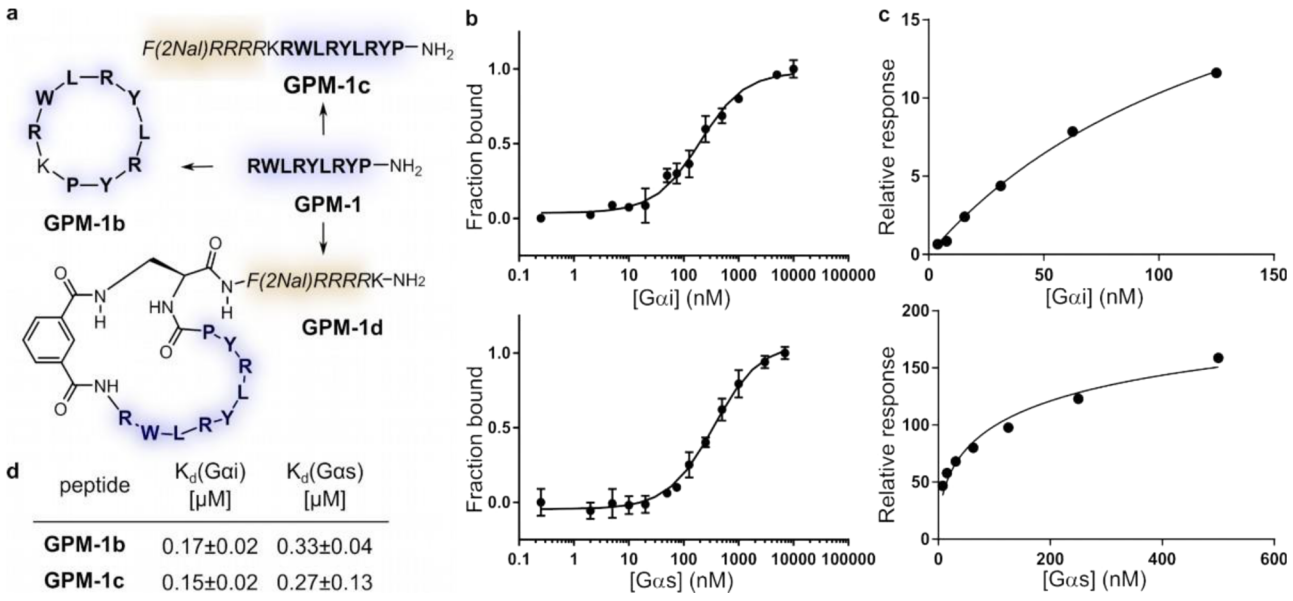

Figure 2. Optimization of GPM-1 and binding results for the derived peptides. (a) **GPM-1b** is the head-to-tail cyclized **GPM-1** (H-RWLRYLRYP-NH_2, blue); **GPM-1c** comprises **GPM-1** and the CPP moiety [F(2Nal)RRRR, yellow]; **GPM-1d** includes the cyclic **GPM-1**(**b**) and the CPP. (b) MST data of FITC-labeled **GPM-1b** with Gαi1/s, error bars represent SD for $n = 3$. (c) SPR data of immobilized biotinylated **GPM-1c** with Gαi1/s, $n = 1$. (d) Obtained K_d values of **GPM-1b** and **GPM-1c**.

and Figure S2). In addition, fluorescein-labeled KB-752 was used as a positive control (Tables S3 and S4).[21] Four of the peptides (**1**, **2**, **7**, and **10**) showed potent binding to Gαi1·GDP (K_d = 140−230 nM) but no or only weak binding to Gαs·GDP (Figures 1a−d and S3). Under the same condition, KB-752 showed a K_d value of 345 ± 40 nM for Gαi and no binding to Gαs, which is somewhat different from the previously reported K_d values of 3900 nM (for Gαi) and 5100 nM (for Gαs) as determined by surface plasmon resonance (SPR, Figure S3).[21] Compared to the previously reported peptide ligands,[14−21] our peptides contain a similar number of hydrophobic aromatic amino acids but a higher percentage of basic (and less acidic) residues (Figure 1d). Peptides **1** and **2** contain a ζWΦ[+/−]ΩΦ-motif (ζ: polar; Φ: hydrophobic; Ω: aromatic amino acid[24]), which is also shared by KB-752, GSP, R6A and R6A-1, AR6-05, cycGiBP, cycPRP-1, cycPRP-3, and Gα SUPR (Figure 1d and Table S2).[3,12−19] Moreover, the binding motif exhibits similarities to the GEM motif in proteins (ΦTΦX[D/E]FΦ).[25−27] In comparison to the alignment of the library-derived peptides (ΦζWΦ[+/−]ΩΦ-motif), the alignment of peptide **1** with the GEM motif can be slightly adjusted to a ΦζΦX[+/−]ΩL-consensus. The sequence similarity suggests that like the previously described peptides and GEM proteins, peptides **1** and **2** may also bind to the Switch II/α3 region of Gαi1.[18,19,21,26,28,32] To test this hypothesis, we examined the ability of peptide **1** to compete with fluorescein-labeled KB-752 for binding to Gαi1 by MST (Figure S4). Peptide **1** indeed inhibited KB-752 for binding to Gαi1 with an IC_{50} value of 155 ± 10 nM. Since Phe8 of KB-752[21] and Phe168 of the GIV−GEM motif[28,33] are crucial for Gαi binding and the GEM activity, we next replaced the corresponding residue of peptide **1** (Tyr5) with an alanine and tested the resulting variant (**1Y5A**) for binding to Gαi1 and Gαs (Tables S3 and S4). **1Y5A** exhibited ∼2-fold lower affinity for Gαi1 than peptide **1** (K_d = 285 ± 40 nM) and no binding to Gαs (Figure S5). Taken together, our data suggest that peptide **1** binds to the Switch II/α3 region of Gαi1 and may function similarly to KB-752 and GEM proteins.[21,28,33] On the other hand, peptides **7** and **10** have lower sequence similarity to the ζWΦ[+/−]ΩΦ-motif in peptides **1** and **2** or to the previously reported peptides and their binding sites on Gαi1 are currently unclear (Figure 1d and Table S2).

Optimization of Hit Peptide. Linear peptide **1**, referred to as **GPM-1** (for "G protein modulator-1", Figures 1, 2, and S6) hereafter, is likely proteolytically unstable and lacks membrane permeability. We thus undertook a limited medicinal chemistry campaign to improve its "drug-like" properties.

First, head-to-tail cyclization of **GPM-1** resulted in **GPM-1b**, which is expected to have improved metabolic stability (Figures 2a and S6, Table S3). Next, a CPP sequence, F(2Nal)RRRR (where 2Nal is L-2-naphthylalanine), and a lysine were added to the N-terminus of **GPM-1** to facilitate cellular entry and fluorescent labeling, respectively, yielding linear peptide **GPM-1c** (Figures 2a and S6, Table S3). This CPP sequence has previously been shown to effectively deliver biologically active peptidyl cargos, e.g., inhibitors against the monomeric G protein K-Ras, into mammalian cells.[34] Its small size also helps to keep the molecular weight of the final construct to a minimum. Finally, **GPM-1** was extended at the C-terminus with a (*S*)-2,3-diaminopropionyl (Dap) unit followed by the CPP motif and subsequently cyclized between its N-terminus and the sidechain amine of the Dap residue by using a bifunctional linker, isophthalic acid (Ipa), to give cyclic peptide **GPM-1d** (Figures 2a and S6, Table S3).

Two control peptides were also prepared. Peptide **14** (H-KRWLRYLRYP-NH_2) serves as a control to assess the effect of the inserted Lys on target binding and the biological activity of **GPM-1**, while peptide **15** reveals whether the CPP sequence alone has any biological activity (Figures 2a and S6, Table S3).

Peptides **GPM-1b−d**, **14**, and **15** were fluorescently labeled and tested for binding to Gαi1/s by MST (Figures 2b and S3, Table S4). As expected, peptide **14** exhibited a similar binding affinity for Gαi (K_d = 140 ± 30 nM) to **GPM-1**, but a lower

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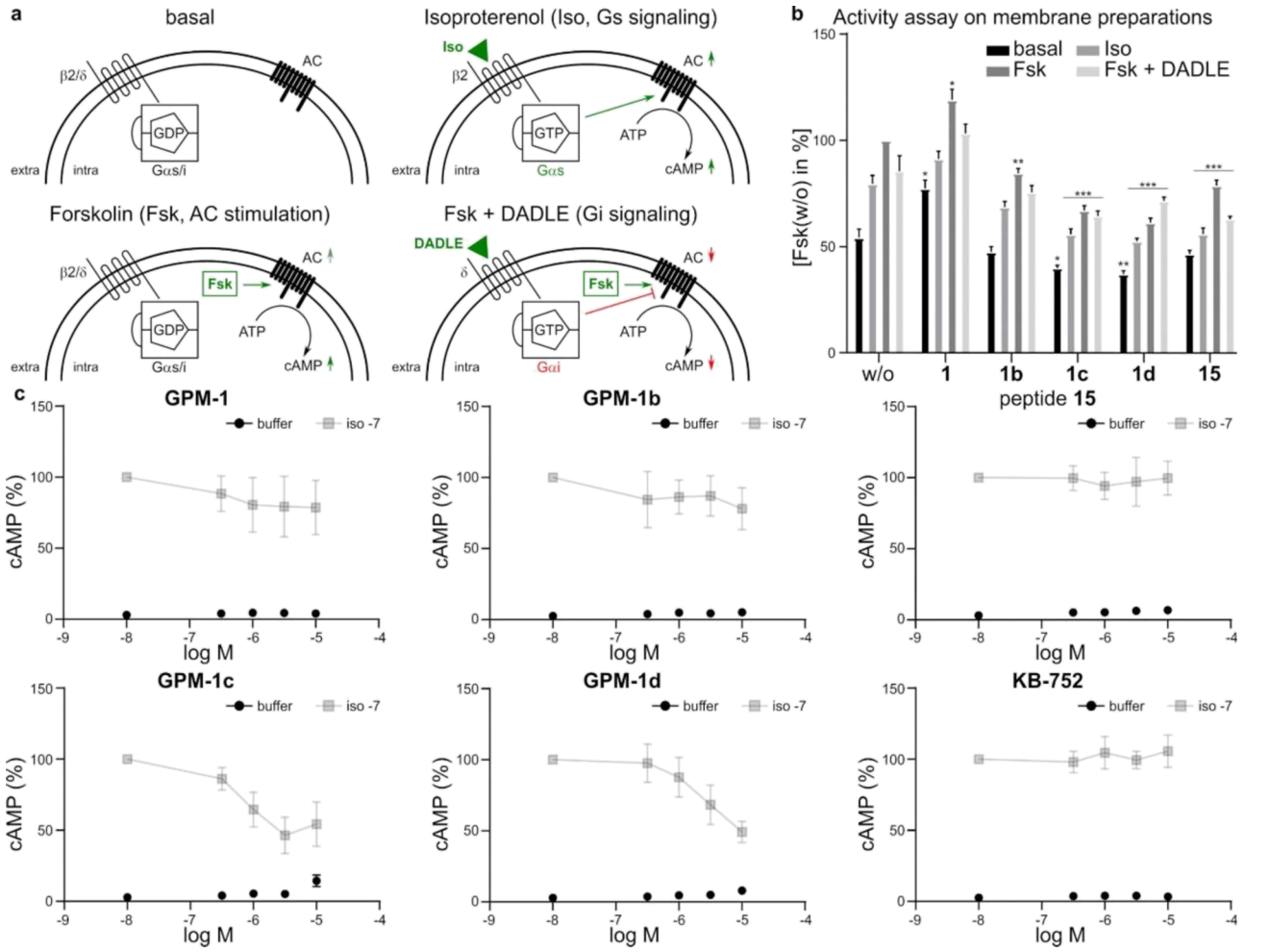

Figure 3. Functional studies of **GPM-1** and its derivatives. (a) Schematic representation of the individual states in the activity studies on NG108-15 membrane preparations. Basal: GPCR (β2 or δ) available for ligand binding, Gα activity is measured independently of the GPCRs. Isoproterenol (Iso) binds to the β2-adrenergic receptor and activates the Gs signaling. Activated Gs stimulates AC leading to an increased cAMP level. Forskolin (Fsk) stimulates AC and increases the cAMP level. Fsk + DADLE: Fsk stimulates cAMP production by direct activation of AC. DADLE ([Tyr-D-Ala^2-Gly-Phe-D-Leu^5]Enkephalin) binds to the δ-opioid receptor and activates the Gi signaling. Activated Gi binds to AC and inhibits the Fsk-stimulated cAMP production. (b) Relative cAMP levels on differently induced membrane preparations incubated with or without the modulators (**GPM-1**, **GPM-1b−d**, **15**) in the presence of a phosphodiesterase inhibitor. cAMP levels were normalized to Fsk or w/o. Shown are percentage values of membranes incubated in the absence (basal), or in the presence of Iso, Fsk and Fsk + DADLE. Error bars represent SD for $n = 3$. Statistical analysis was performed using the one-way ANOVA test Dunnet corrected, with $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$ for comparisons with the control (w/o). (c) Iso-induced cAMP accumulation in HEK293 cells. Depicted are the buffer controls (black) and the Iso values (gray) for **GPM-1**, **GPM-1b−d**, **15** and KB-752 in % cAMP. Error bars represent SD for $n = 3$. None of the peptides was found to influence the vitality of the cells in an MTT assay on HEK293 cells at a maximum concentration of 10 μM.

binding affinity for Gαs (K_d = 1010 ± 160 nM). Cyclization of **GPM-1** slightly decreased its binding affinity for Gαi (K_d = 170 ± 20 nM for **GPM-1b**, Figure 2b,d), but unexpectedly increased the affinity for Gαs (K_d = 330 ± 40 nM for **GPM-1b**). The CPP-containing peptides (**GPM-1c**, **GPM-1d**, and **15**) could not be analyzed by MST, presumably because the positively charged sequences resulted in strong binding of the peptides to the negatively charged glass wall of the capillaries.[35] We therefore labeled peptides **GPM-1**, **GPM-1c**, **GPM-1d**, and **15** with a biotin (for surface immobilization, Table S4) and analyzed them for binding to Gαi1·GDP and Gαs·GDP by SPR (Figures 2c and S7). Peptides **GPM-1**, **GPM-1c**, and **15** bound to Gαi1 with K_d values of 170 ± 50 nM, 150 ± 20 nM, and 530 ± 70 nM, respectively (Figures 2c,d and S7). Thus, for **GPM-1**, there is good agreement between the K_d values derived from MST and SPR assays. As expected, **GPM-1c** retained the binding affinity of **GPM-1** for Gαi1·GDP. Surprisingly, peptide **15** (the CPP moiety alone) showed significant binding affinity for Gαi1, although the affinity is considerably lower than that of **GPM-1** and **GPM-1c**. Note that peptides **15** and **GPM-1** share substantial structural similarities, in that they are both rich in arginine and aromatic hydrophobic residues. We were not able to reliably determine the binding affinity of **GPM-1d** by SPR, because protein binding to the immobilized peptide resulted in only small response unit (RU) changes. **GPM-1d** was directly used in the activity studies, as we expected it to have similar binding affinity to **GPM-1b** and **GPM-1c**, which are both high-affinity Gαi binders. Importantly, **GPM-1** (K_d = 560 ± 60 nM) and **GPM-1c** (K_d = 270 ± 130 nM) showed weaker binding to Gαs than Gαi1, in agreement with the MST data for **GPM-1** (K_d = 630 ± 90 nM). Peptide **15** displayed substantially lower affinity for Gαs (K_d = 2000 ± 720 nM, Figure S7).

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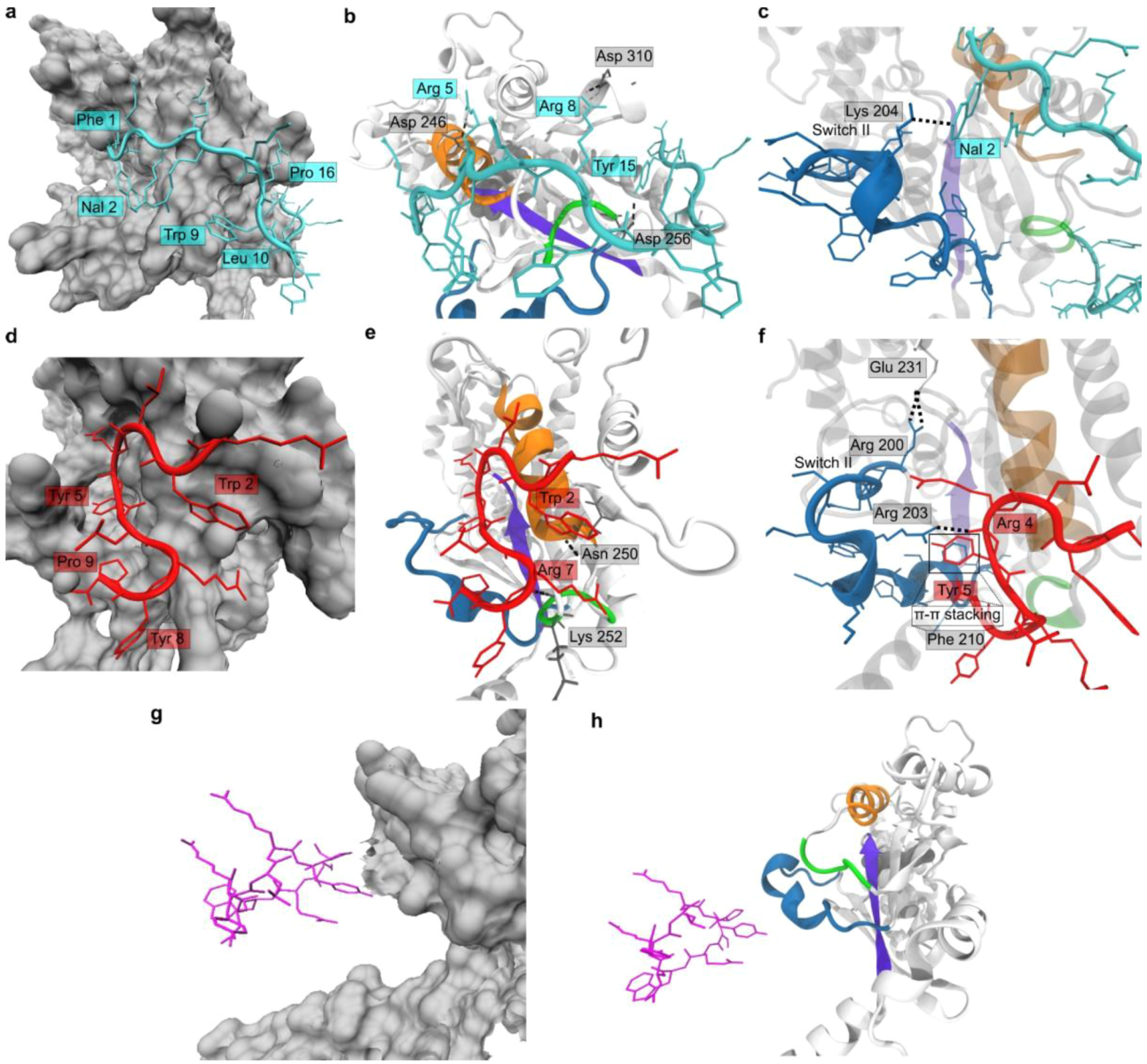

Figure 4. Computational studies of Gαi-peptide interactions. (a,d,g) Molecular surface (gray) of Gαi on which **GPM-1c** (cyan), **GPM-1** (red), and **GPM-1b** (magenta) are bound. In (a) and (d), the side chains involved in hydrophobic interactions with Gαi are labeled. (b,e,h) Gαi structure (white cartoon) with Switch II (blue), α3 (orange), β1 (violet), and α3−β5 loop (green) depicted. The bound conformation of **GPM-1c** (cyan), **GPM-1** (red), and **GPM-1b** (magenta) are presented, with the H-bonding (black dotted lines) partners labeled (for **GPM-1c**, **GPM-1**). (c,f) Closer look at the interactions between Switch II (blue) of Gαi and **GPM-1c** (cyan) and **GPM-1** (red). H-bonding interactions are shown as black dotted lines, the residues involved are labeled. In (f), the π−π stacking interactions between Tyr5 of **GPM-1** and Phe210 of Switch II are highlighted in a square.

Biological Activity in Cell Culture. The biological activity of **GPM-1** and its derivatives was assessed by two different assays. In a cell-free ELISA-based assay,[36] membrane-bound inhibitory (δ-opioid) and stimulatory (β2-adrenergic) GPCRs converge at the level of AC, allowing the use of cAMP production as a common readout (Figure 3a,b). Regulation of the Gs function can be assessed under basal, isoproterenol (Iso; receptor activation)- and forskolin (Fsk; direct activation of AC)-stimulated conditions, whereas any regulatory effect on Gi is gleaned from the inhibition of Fsk-stimulated cAMP production by [Tyr-D-Ala^2-Gly-Phe-D-Leu^5]Enkephalin (DADLE). A whole-cell assay was also established with HEK293 cells overexpressing the SNAP-β2 adrenergic receptor to specifically monitor the Gs signaling pathway after stimulation with Iso in a concentration-dependent manner (Figure 3c). It should be noted that since the change in cAMP levels represent the sum of all G protein activities, these assays only detect major regulatory effects on dominant signaling cascades.

Among the peptides that bound to Gαi1 or Gαs in the biochemical tests (peptides **GPM-1**, **2**, **7**, and **10**), only **GPM-1** and its derivatives exhibited significant biological activity in the cell-free or whole-cell assay. In addition to our peptides, KB-752 was included for comparisons as no data from whole cell assays could be retrieved from the literature.[21−23]

In the cell-free assay, **GPM-1** resulted in an overall increase in the basal, Iso- and Fsk-stimulated cAMP production, relative to the vehicle control (w/o) (Figure 3b). At the meantime,

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GPM-1 reduced the stimulatory effect of Iso by ∼10%. In the cell-based assay, **GPM-1** also decreased the Iso-mediated cAMP production in a concentration-dependent manner, by ∼20% at 10 μM peptide concentration (Figure 3c). The cellular activity of **GPM-1** may seem surprising, because it is not conjugated to any CPP; however, **GPM-1** contains three arginine residues as well as several hydrophobic residues and likely possesses significant cell-penetrating activity. Interestingly, KB-752 has previously been reported to inhibit Gs signaling in a membrane preparation assay,[22] which was confirmed in our ELISA experiment (Figure S8). However, KB-752 has no effect on Gαs in the whole cell assay (Figure 3c). The simplest explanation of these observations is that both KB-752 and **GPM-1** act as GDI toward Gαs.[22,27] Indeed, since **GPM-1** shares a high sequence similarity to KB-752 within the Gαi/s-binding motif, a similar mechanism of action is not unexpected. Lack of activity in the whole-cell assay by KB-752 is likely because the latter contains multiple negatively charged residues in its sequence and is impermeable to the cell membrane. **GPM-1 Y5A** showed no effect in the ELISA (Figure S8), which further corroborates the KB-752-like effect of **GPM-1**, as Phe8 in KB-752 is critical for the GEM activity.[21,28,33] As expected, peptide **14** behaved very similar to **GPM-1** (Figure S9), whereas **GPM-1b** showed no functional regulation of Gαs (Figure 3b). The latter is in good agreement with the result of the whole cell assay (Figure 3c) and could be a consequence of the higher rigidity of the cyclic structure compared to **GPM-1** despite of its binding affinity.

In the cell-free assay, the CPP-linked peptides **GPM-1c** and **GPM-1d** produced an overall reduction in the basal, Iso- and Fsk-stimulated cAMP levels (Figure 3b). This effect was much greater in the cell-based assay; both **GPM-1c** and **GPM-1d** dose-dependently reduced Iso-stimulated cAMP production, causing ≥50% inhibition at 10 μM (Figure 3c). The CPP alone (peptide **15**) was inactive in the whole cell assay, suggesting that the observed effect in the ELISA-based assay is likely caused by nonspecific interactions (Figure 3b,c). These data indicate that **GPM-1c** and **GPM-1d** are more effective GDIs than **GPM-1**.

With respect to Gi signaling, while **GPM-1**, **GPM-1b**, **GPM-1**, **Y5A** (as well as peptide **15** and KB-752) showed no effect on the Fsk-stimulated AC activity, **GPM-1c** and **GPM-1d** increased Gi signaling by 19 and 22%, respectively, relative to the control (w/o) (Figure 3b). Additionally, while **GPM-1**, **GPM-1b**, **GPM-1 Y5A**, KB-752, and peptide **15** had no effect on DADLE-mediated inhibition of the AC activity, **GPM-1c** almost completely abolished the effect of DADLE and **GPM-1d** increased the AC activity by ∼10% after activation of inhibitory δ-opioid receptors with DADLE (Figure 3b). These results suggest that **GPM-1c** and **GPM-1d** function as Gαi-specific GEFs resulting in permanent activation of Gαi as described previously for KB-752.[21,22]

Taken together, the above data demonstrate that **GPM-1c** and **GPM-1d** bind to and regulate the function of both Gi (as GEF) and Gs (as GDI) thus acting as bifunctional GEMs, as previously demonstrated for KB-752[21,22,27] and GEM proteins.[25−27] However, while KB-752 and GEM proteins are impermeable to the cell membrane, **GPM-1c** and **GPM-1d** are cell-permeable and biologically active in whole-cell assays. Among the two peptides, **GPM-1d** is the preferred ligand, because of its higher potency in the cellular assay as well as greater proteolytic stability (thanks to its cyclic structure).

Computational Analysis of Gαi1/s−Peptide Complexes. To gain insight into the structural basis for the observed biological activities, we carried out a series of computational analyses for the interaction of Gαi/s with the peptide modulators experimentally deemed to be active (**GPM-1c**, **GPM-1d**), slightly active (**GPM-1**), and inactive (**GPM-1b**, **GPM-1 Y5A**, **15**). We describe briefly below the Gαi/s−peptide interactions observed from 50 ns MD trajectories of docked Gαi/s−peptide complexes and the structural and energetic implications. Additional details such as the Gαi homology model, determination of Gαi/s binding site, and molecular docking to Gαi/s are provided in the SI (Figures 4 and S10−S17, Tables S5−S10).

GPM-1c binds to a region around Switch II, α3, and β1 of Gαi (Figures 4a−c and S10, Video S3) through hydrophobic interactions between the side chains of Phe1, Nal2, Trp9, Leu10, and Pro16 of **GPM-1c** and binding sites 2 and 3 (Figure S12) on Gαi. Additional binding energy as well as specificity are derived from persistent **GPM-1c**-Gαi hydrogen (H)-bonding interactions between Arg5-Asp246, Arg8-Asp310, Tyr15-Asp256, and 2Nal-Lys204 (Switch II), which were observed for >80% of the simulation (Figure 4c). This renders the structure of the bound peptide stable upon Gαi binding with a backbone RMSD of 3.21 ± 0.81 Å relative to its initial conformation (Table S10).

GPM-1d and Gαi engage in a network of H-bonding interactions between Arg14-Asp246, Arg14-Asn250, Arg16-Asn251, Arg14-Lys252, and Arg4-Glu211 (Switch II) in >70% of the simulation. The complex is also stabilized by hydrophobic interactions between Trp2 of **GPM-1d** and binding site 3 on the Gαi surface (Figures S12 and S16) as well as an intramolecular stacking interaction between the side chain of Tyr8 and N-terminal Ipa. It appears that cyclization of **GPM-1d** enhances protein binding by constraining it into the binding conformation (Table S10). On the other hand, cyclization prevented the peptide side chains from adopting optimal interactions with the Gαi surface, resulting in a reduction of its computed binding affinity.

GPM-1 showed a much lower binding affinity (−300.23 kJ mol^{-1}, Table S11) than **GPM-1c** and **GPM-1d**. **GPM-1** bound similarly to KB-752[21] and the GIV GEM motif[26,28] around α3 and the α3−β5 loop of Gαi (Figures 4d−f, S10, and S11 and Video S1). The side chains of Trp2, Tyr5, Tyr8, and Pro9 are largely buried into the hydrophobic groves of the binding site (Figure 4d), with **GPM-1**-Gαi H-bonding interactions between Trp2-Asn250, Arg7-Lys252, and Arg4-Arg203 (Switch II, Figure 4e,f) providing additional stabilization. A π−π stacking interaction between Phe210 (Switch II) and Tyr5 of **GPM-1** add to the stability as previously described for GEMs at the respective position (Figures 4f and 5).[28] Switch II residues (including Phe210) are involved in G$\beta\gamma$ binding, explaining why the binding of **GPM-1** and G$\beta\gamma$ to Gαi might be mutually exclusive.[28,33]

The inactivity of **GPM-1b**, **GPM-1 Y5A**, and peptide **15** can be explained from the MD simulations of their bound conformations on Gαi. **GPM-1 Y5A**, adopting almost a cyclic conformation upon folding (Figure S15), bound poorly to Gαi (Figure S16 and Table S11), reflecting its inactive nature in the experiments. This indicates that the interaction of Tyr5-Phe210 is important for the Gαi binding of **GPM-1**, which was also described for GEMs.[23,28] The cyclic **GPM-1b** moves away from its bound conformation into solution during the simulation and is held in the vicinity of the protein only via

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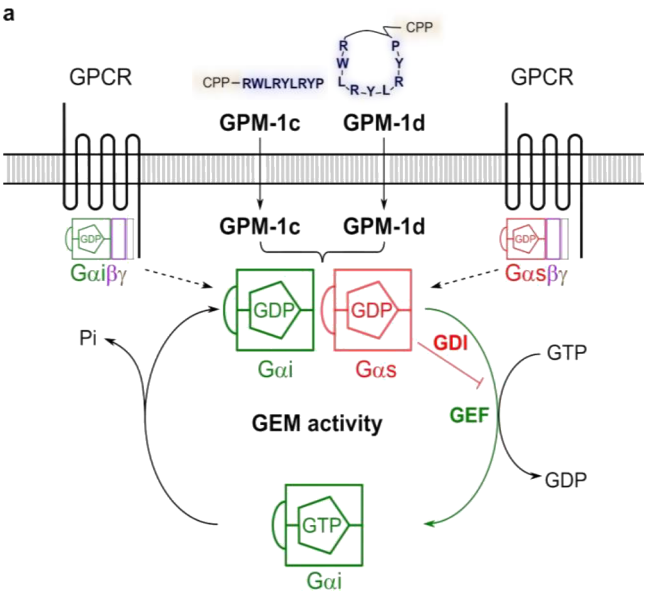

b

		Φ ζ Φ X[+/-] Ω L	
KB-752	- - - - - - - S R	V T W Y D F L	M E D T K S R
GSP	M A M S D R N K R	L T V W E F L	A L P S S T -
GPM-1	- - - - - - - - -	- R W L R Y L	R Y P - - - -
PLCδ4b	- - - - - - - Q K	L T L L E F L	D F L Q E E Q
GIV	- - - - - - - E V	V T L Q Q F L	E E S N K L T
DAPLE	- - - - - - - E M	V T L E E F L	E E S N R S S
GBAS-1	- - - - - - - E T	V T V E E F L	M N S Y S - -
Calnuc	- - - - - - - R L	V T L E E F L	A S T Q R K E
NUCB2	- - - - - - - R L	V T L E E F L	K A T E K K E

Figure 5. GEM-like effect of **GPM-1c** and **GPM-1d**. (a) Schematic representation of the GEM activity of **GPM-1c** and **GPM-1d**, which are able to penetrate the cell membrane. Inside the cell, both can cause a GEF effect on Gαi and a GDI effect on Gαs, thus reducing the overall cAMP production. (b) Sequence alignment of **GPM-1** with KB-752,[21] GSP[17] and the GEM motifs of the GEM proteins.[25−27] Symbols as follows:[24] Φ: hydrophobic (V, I, L, F, W, Y, M), Ω: aromatic (F, W, Y), ζ: uncharged hydrophilic (N, Q, S, T), [+]: basic (H, K, R) and [−]: acidic (D,E) amino acids.

long-range nonbonded interactions (Figure 4g,h, Video S2). Peptide **15** also moved away from its Gαi-bound conformation and remained in this unbound state throughout, i.e., peptide **15** also had a poor binding energy (Table S11).

An interesting observation was made with the binding energies computed between the Gαi·GDP protein with and without the peptides. It appears that the quality of peptide binding to Gαi is anticorrelated with the binding of GDP. Gαi·GDP had a MMPBSA binding energy of −306.23 kJ mol^{-1}, which was considered as the reference value for the GDP association. With **GPM-1c** and **GPM-1d** bound, the average binding energy of GDP to Gαi decreased to −436.38 and −433.67 kJ mol^{-1}, respectively (Table S11 and Figure S18). With the moderately active **GPM-1** bound, the binding energy of GDP was 286.27 kJ mol^{-1}, while with **GPM-1 Y5A**, **GPM-1b**, and peptide **15**, the GDP binding energies were 158.65, 318.34, and 309.67 kJ mol^{-1}, respectively (Table S11). This observation suggests a tantalizing possibility that binding by the peptides may promote GDP release from Gαi (i.e., nucleotide exchange). This is consistent with the experimental results, which suggest **GPM-1c** and **GPM-1d** as GEF for Gαi.[28]

Conversely, the binding of **GPM-1**, **GPM-1c**, and **GPM-1d** on Gαs (Figures S17 and S18) enhances GDP binding to Gαs. All three peptides bound on the predicted binding site 2 (Figures S13 and S17) between Switch II and α3 and provide a direct cover for the GDP molecule. Direct H-bonded interactions between the peptides and GDP were observed. The peptides exhibited higher MMPBSA binding energies in comparison to their interactions with Gαi, and improved binding of GDP to Gαs, indicating a GDI-like action (Table S12). **GPM-1Y5A**, which bound to a different region between binding sites 2 and 3, had a poor binding affinity.

Advantages over Current Gαi/s Modulators. Direct targeting of G proteins provides an attractive alternative to GPCR modulators for treating many human diseases, such as cancer.[2,3,5−7] However, modulators that are capable of binding reversibly and specifically to Gαi/s have been challenging to develop.[2,3] The widely used protein modulators, such as CTX and PTX, covalently and irreversibly modify the G proteins,[3,10,11] while KB-752 is impermeable to the cell membrane and cannot be used in cellular or in vivo assays. **GPM-1c** and **GPM-1d** have a high potential of pharmacological significance, because they possess GIV-like GEM activity, are able to occupy the Gαi−GIV interface and thus might influence the (GIV-mediated) Gαi activity.[3,28,37] Furthermore, the peptides can affect the G protein activity independently of the GPCRs, which can provide insights into the G protein-mediated signaling and related diseases and bypasses the need to address individual receptors in disorders directed by multiple GPCRs.

CONCLUSIONS

In this work we discovered linear (**GPM-1c**) and cyclic peptides (**GPM-1d**) as a novel class of cell-permeable, Gαi/s-selective, and reversible modulators of Gα protein activity (Figure 5). These Gαi/s GEMs appear to bind to the Switch II/α3 region, as do KB-752 and the GEM proteins,[25−28] and may thus affect Gαi/s downstream signaling and the resulting cellular response. Since G proteins are generally "undruggable"[3,38] and have a crucial role in the pathogenesis of cancer, **GPM-1c** and **GPM-1d** should serve as valuable chemical tools in pharmacological research and potential leads for further development into therapeutic agents to finally achieve druggability for Gs and Gi.[3,5,38] This study also demonstrates that a combination of peptide library screening and medicinal chemistry offers a viable approach to developing novel Gα modulators.

METHODS

Expression and Purification of Gαi1/s Protein. Transformed *E. coli* BL21 (DE3) cells were used that contained a vector construct of the plasmid pET28a (+) and the DNA sequence encoding for a hexahistidine tag and an enterokinase cleavage site [Met-Gly-Ser-Ser-$(His)_6$-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Ser-(Asp)4-Lys] followed by the sequence encoding for Gαi1 (Uniprot ID: P63096), where the initial methionine was deleted according to Suzuki et al.[39] The same procedure was followed for Gαs (short isoform, Uniprot ID: P63092-2).[40] Expression of Gαi1 was modified from Chen et al.[41] The His-tagged protein was purified in a slightly modified approach according to Tesmer et al.,[42] whereas Gαs protein preparation was performed as described earlier introducing modifications to these protocols.[40−44] Protein samples (Gαi1 in 20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 250 mM imidazole, 10 mM β-mercaptoethanol, 50 μM GDP, 10% (v/v) glycerol and Gαs in20 mM HEPES pH 8.0, 100 mM

https://doi.org/10.1021/acschembio.1c00929
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NaCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, 50 μM GDP, 10% (v/v) glycerol) were shock-frozen and stored at −80 °C.

Biotinylation of the Gαi1 Protein for Library Screening. NHS-biotin (2.5 equiv) in DMSO was added to the Gαi1 protein (1 equiv) and carefully stirred at room temperature for 30 min. Then, Tris buffer (2 M, pH 8.15) was added and the solution was stirred for 10 min. The biotinylated G protein was purified using a PD-10 column (SephadexTM G-25M with 0.1% KathonTM CG, GE Healthcare), and the protein concentration was determined by Bradford assay according to the manufacturer's instruction (ROTI Nanoquant, Carl Roth GmbH + Co. KG).[45] The Gαi1 protein solution was shock-frozen with 10% (v/v) glycerol and stored at −80 °C until usage.

OBOC Library Screening. The screening of the peptide library (X4[C/H/Y]0X420, where X is any of the proteinogenic amino acids and Nle, excluding Cys and Met), the hit selection, and identification was performed as earlier described.[30,31]

Peptide Synthesis and Purification. Solid-phase peptide synthesis according to the Fmoc strategy was performed on a Rink amide MBHA resin (0.53 mmol g^{-1}) or a 2-chlorotrityl resin (KB-752: 0.40 mmol g^{-1}, GPM-1b: 0.70 mmol g^{-1}). Hits from the library screening (GPM-1, 2−13), Peptide 14, GPM-1 Y5A, and KB-752 were synthesized with an automated ResPep SL peptide synthesizer from Intavis Bioanalytical Instruments GmbH using HBTU (4 equiv) as the coupling reagent and *N*-methylmorpholine (NMM, 9 equiv) as the base. The optimized hits were synthesized manually with HBTU (4 equiv) and HOBt (4 equiv, GPM-1b, GPM-1c, 15) or HATU (4 equiv, GPM-1d) as coupling reagents and DIPEA (8 equiv) or NMM (8 equiv) as base. The cleavage of the side-chain-protecting groups together with the peptide from the resin was performed with reagent K as described previously.[29] For GPM-1b, the linear sequence of GPM-1 was synthesized on the 2-chlorotrityl resin and, subsequently, Boc-Lys(Fmoc)-OH was coupled to the N-terminus. After cleavage of the linear precursor from the resin, the peptide was cyclized in solution with PyBOP (6 equiv) and DIPEA (12 equiv). The subsequent Fmoc group removal was achieved using diethylamine (20 equiv) in DMF. For GPM-1d, first Fmoc-Lys(Boc)-OH and the CPP sequence (F(2-Nal)RRRR) by using Fmoc-Phe-OH, Fmoc-(2-Nal)-OH, and Fmoc-Arg(Pbf)-OH were coupled to the resin, and then, Fmoc-Dap(Alloc)-OH was coupled according to Lian et al.[34] for later cyclization. Thereafter, the peptide was extended by the GPM-1 sequence (RWLRYLRYP) by using standard Fmoc strategy. Subsequently, isophthalic acid was coupled and the Alloc group was cleaved by $Pd(PPh_3)_4$ (0.5 equiv) and phenylsilane (10 equiv) in DCM. After cyclization with PyBOP (10 equiv), HOBt (10 equiv) and NMM (20 equiv) on the resin, the peptide was cleaved from the resin with reagent K as described previously.[29] The crude peptides were purified by semi-preparative reversed-phase HPLC using a Shimadzu LC-8A instrument equipped with a Knauer Eurospher column (C18, 250 × 32 mm, 5 μm particle size, 100 Å pore size) for amounts between 20−80 mg or a Vydac 218TP1022 column (C18, 250 × 22 mm, 5 μm particle size, 100 Å pore size) for amounts up to 20 mg with a gradient of 0.1% (v/v) TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (90:10, eluent B). For each peptide, a gradient was selected according to the elution behavior in the analytical RP-HPLC by increasing 50% eluent B at a flow rate of 10 mL min^{-1} in 120 min. The peaks were detected at 220 nm. The purity of the peptides (>98%) was confirmed by analytical RP-HPLC from a Shimadzu LC-20AD system equipped with a Vydac 218TP column (C18, 250 × 4.6 mm, 5 μm particle size, 300 Å pore size) with a gradient system of 0.1% TFA in water (eluent A) and 0.1% (v/v) TFA in acetonitrile (eluent B) at a flow rate of 1 mL min^{-1} and 220 nm detection. The collected fractions were combined, freeze-dried, and stored at −20 °C. Detailed information on the individual peptides can be found in Supporting Information (Table S3).

Fluorescence Labeling of Peptides. All linear peptides were N-terminally labeled on resin with 5(6)-carboxyfluorescein (Cf, 2 equiv) in DMF using PyBOP (2 equiv) as coupling reagent and DIPEA (3 equiv) as base. All cyclized peptides were selectively labeled with fluorescein isothiocyanate (FITC) in solution according to Trinh et al.[34] on the side chain of the lysine previously inserted into the sequence. Therefore, after dissolving the purified peptide (1.5 mg) in DMSO (34 μL) and 100 mM $NaHCO_3$ pH 8.5 (34 μL), FITC (15 μL, 10 mg mL^{-1} in DMSO) was added, and the solution was incubated at 25 °C for 40 min in the dark. Then, 50% (v/v) TFA/water (7.5 μL) was added, and the labeled peptide was purified by reversed-phase HPLC using a Shimadzu LC-10AT system equipped with a Vydac 218TP column (C18, 250 × 4.6 mm, 5 μm particle size, 300 Å pore size), and a mobile phase system consisting of 0.1% (v/v) TFA in water (eluent A) and 0.1% (v/v) TFA in acetonitrile (eluent B). The purity of the peptides (>98%) was confirmed by analytical RP-HPLC and the identity of the peptides was validated with mass spectrometry. Detailed information on the fluorescence-labeled peptides can be found in Table S4.

Synthesis of Biotinylated Peptides. For the peptides GPM-1 and 15, biotinylated analogues, Btn-GPM-1 and Btn-15, were synthesized on a Rink amide MBHA resin (0.53 mmol g^{-1}) by solid-phase peptide synthesis according to Fmoc strategy using an automated ResPep SL peptide synthesizer (Intavis Bioanalytical Instruments GmbH). First, Fmoc-Lys(Biotin)-OH and Fmoc-O_2Oc-OH and then the corresponding sequence (GPM-1: RWLRYLRY; 15: F(2-Nal)RRRR) were coupled with HBTU (4 equiv) as coupling reagent and *N*-methylmorpholine (9 equiv) as base. The cleavage of the side chain protecting groups and the peptides from the resin took place as described before.[29] GPM-1c and GPM-1d were biotinylated in solution with NHS-PEG_4-Biotin (Thermo Fisher Scientific Inc.) at the lysine side chain. After the peptide (1−2 mg) was dissolved in DMSO (50 μL) and phosphate buffer (950 μL, 50 mM, pH 6.5), NHS-PEG_4-Biotin (0.5 equiv for GPM-1c, 5 equiv for GPM-1d, 10 mM in DMSO) was added and the reaction was incubated for 90 min at 4 °C. The biotinylated peptides, Btn-GPM-1c and Btn-GPM-1d, were purified by reversed-phase HPLC using a Shimadzu LC-10AT instrument as described above. Subsequently, the correct biotinylation pattern of GPM-1c was determined by automated Edman degradation. The N-terminal sequence analysis was performed using a Shimdazu PPSQ-53A protein sequencer. Prior to analysis, the peptide was dried under vacuum, freshly dissolved in 0.5% (v/v) acetic acid, and 10 pmol of the peptide were applied to a polybrene treated Glass Fiber Disk. After drying under a stream of nitrogen for 10 min, 2 cycles of N-terminal sequence analysis were performed. The derivatized amino acids were separated isocratically by RP-HPLC on a Shimadzu LC-20AT with a Wakopak Wakosil PTH-II column (C18, 4.6 mm × 250 mm, 5 μm particle size), detected at 269 nm, and identified by comparison with a PTH-standard mixture. The non- and double-biotinylated analogues were also examined in the same way. The purity of the peptides (>98%) was confirmed by analytical RP-HPLC and the identity of the peptides was validated with mass spectrometry. Detailed information about the biotinylated peptides can be found in Table S4.

Peptide Analysis. The peptides were characterized by analytical RP-HPLC (see above) and mass spectrometry. The characterization of the peptides by mass spectrometry was performed as described previously.[46] GPM-1 and peptides 2−13 as well as Cf-GPM-1 and peptides Cf-2−13 were additionally analyzed by amino acid analysis as reported earlier.[46] In addition, the peptide content of all other peptides was determined with a GPM-1 or Cf-GPM-1 calibration curve by analytical RP-HPLC using the GPM-1 or Cf-GPM-1 peptide content from amino acid analysis as a basis. Detailed information on the individual peptides can be found in Tables S3 and S4.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00929.

Experimental procedures and instrumentation used, methods for binding and activity studies, including characterization data (Figures S1−S18), computational

https://doi.org/10.1021/acschembio.1c00929
ACS Chem. Biol. 2022, 17, 463−473

analysis of protein−peptide interactions and corresponding data (Tables S1−S12) (PDF)

Computational analysis of protein−peptide interactions and corresponding data (MP4) (MP4) (MP4)

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Author Contributions

D.I. conceived the research and designed the experimental studies with support of B.N. and T.K.; D.P. provided advice in combinatorial library screening and use of CPPs. B.N. performed the screening, the hit synthesis, purification, and analytical characterization of the peptides as well as the MST and SPR measurements. M.S.B. and B.N. carried out the sequence analysis of the biotinylated peptides. A.P. and T.K. expressed and purified the Gα proteins. B.N. and T.K. conducted the biotinylation of the Gαi1 protein, the PED−MALDI−MS, and consensus sequence analysis. H.A., M.M., and B.N. performed and analyzed the peptide−G protein interactions in the cAMP assays on membrane preparations. E.M.P. and S.K.A. performed the cAMP assays on HEK293 cells. MTT assays were conducted by M.S.B. and B.N.; A.A.P.G. and R.M. carried out and/or analyzed the computational studies. B.N. collected and analyzed the data together with D.I. and D.P. All authors discussed the results and contributed to the final manuscript.

Funding

This work was supported by the University of Bonn, the Deutsche Forschungsgemeinschaft (DFG) within FOR 2372 and IM 97/14-1 (to D.I.), and the Bonner Universitätsstiftung (to B.N.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank E. Kostenis, M. Geyer, and M. Famulok (University of Bonn) for access to cells, assays, and instruments, S. Linden, F. Steinbock, U. Rick (all University of Bonn), G. Schlipf (LMU Munich), and V. Uzunova (Cytiva Europe GmbH) for technical support, K. Hampel (Biaffin GmbH & Co KG) for support with SPR analysis, E. A. Galinski and M. Hecker (University of Bonn) for the collaboration on Gα protein expression, and Solvay GmbH for the friendly supply of chemicals.

ABBREVIATIONS

AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; CPP, cell-penetrating peptide; CTX, cholera toxin; GDI, guanine-nucleotide dissociation inhibitor; GEF, guanine-nucleotide exchange factor; GEM, guanine-nucleotide exchange modulator; GPCR, G protein-coupled receptors; MST, microscale thermophoresis; PTX, pertussis toxin; SPR, surface plasmon resonance

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https://doi.org/10.1021/acschembio.1c00929
ACS Chem. Biol. 2022, 17, 463−473

Appendix E: Bicyclic Peptide Library Screening for the Identification of Gαi Protein Modulators

A. Pepanian, F. A. Binbay, S. Roy, B. Nubbemeyer, A. Koley, C. A. Rhodes, H. Ammer, D. Pei, P. Ghosh, and D. Imhof. Bicyclic peptide library screening for the identification of Gαi protein modulators. (**2023**)

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Bicyclic Peptide Library Screening for the Identification of Gαi Protein Modulators

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Cite This: J. Med. Chem. 2023, 66, 12396−12406

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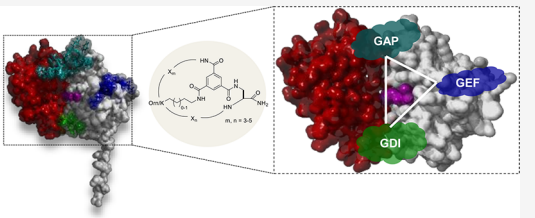
ABSTRACT: Noncanonical G protein activation and inactivation, particularly for the Gαi/s protein subfamilies, have long been a focus of chemical research. Combinatorial libraries were already effectively applied to identify modulators of the guanine-nucleotide exchange, as can be exemplified with peptides such as KB-752 and GPM-1c/d, the so-called guanine-nucleotide exchange modulators. In this study, we identified novel bicyclic peptides from a combinatorial library screening that show prominent properties as molecular switch-on/off modulators of Gαi signaling. Among the series of hits, the exceptional paradigm of GPM-3, a protein and state-specific bicyclic peptide, is the first chemically identified GAP (GTPase-activating protein) modulator with a high binding affinity for Gαi protein. Computational analyses identified and assessed the structure of the bicyclic peptides, novel ligand−protein interaction sites, and their subsequent impact on the nucleotide binding site. This approach can therefore lead the way for the development of efficient chemical biological probes targeting Gαi protein modulation within a cellular context.



INTRODUCTION

The heterotrimeric Gαβγ proteins undoubtedly play an essential role in numerous signaling pathways. G proteins are classified into the four main subfamilies: the Gαs, Gαi/o, Gαq/11, and Gα12/13.[1,2] These proteins are activated by G protein-coupled receptors (GPCRs) and oscillate between the inactive GDP-bound and active GTP-bound state, with the latter interacting with multiple effector proteins, such as the adenylyl cyclase (AC) and phospholipase Cβ via protein−protein interactions (PPIs).[3] Dysregulation or abnormalities of the G protein-mediated signaling pathway can be associated with several diseases, including different cancer types.[4−6] As recently described, the development of G protein modulators as chemical tools is fundamental to obtain further insight into the G protein-mediated signal transduction.[7−10]G proteins have been designated in the past as "undruggable" and challenging targets due to their intracellular localization and the close structural homology between the members of the Gα protein families.[11] In our recent study, we were able to obtain from a one-bead-one-compound (OBOC) combinatorial peptide library screening a promising linear peptide as a lead compound (GPM-1, RWLRYLRYP), which was further optimized by cyclization and conjugation to a cell-penetrating peptide (CPP).[7] Such high-throughput screening (HTS) technologies, including also the one-bead-two-compound (OBTC) libraries and the random nonstandard peptide integrated discovery (RaPID) system,[12−15] have been frequently used for the identification of high-affinity cyclic peptide binders to a variety of proteins. This can be exemplified with the bicyclic cyclorasin B4−27[16] acting as an inhibitor of the monomeric G protein K-Ras. Apart from the possibility to insert unnatural amino acids, in particular, bicyclic peptides may have the advantage of an enhanced rigidity that may lead to an increased metabolic stability and even cell permeability.[7,13,14,17−20] In this way, a variety of macrocyclic peptides that bind to, e.g., the Gαi•GDP protein, have already been identified, such as cycGiBP,[26] cycPRP-1,[27] cycPRP-3,[27] and Gα SUPR peptide,[28] as well as GPM-1b and GPM-1d.[7] Concerning these peptides, no structural investigation has been reported regarding the protein−macrocyclic peptide complex formation for both protein states (active and inactive).[26−28] The aforementioned linear peptide GPM-1 was derived from a peptide library screening against Gαi1·GDP and displayed high similarity to the phage display-derived peptide KB-752.[7,29] The latter one was described to act as a guanine-nucleotide exchange modulator (GEM), i.e., a guanine-nucleotide exchange factor (GEF, Figure 1a) for Gαi and a guanine dissociation inhibitor (GDI) for Gαs,[8] similar to the bifunctional GIV/Girdin, the prototypical member of the GEM family.[30−33] Considering the effectiveness of G protein modulators, however, it is required that the molecules are

Received: May 15, 2023
Published: August 16, 2023

https://doi.org/10.1021/acs.jmedchem.3c00873
J. Med. Chem. 2023, 66, 12396−12406

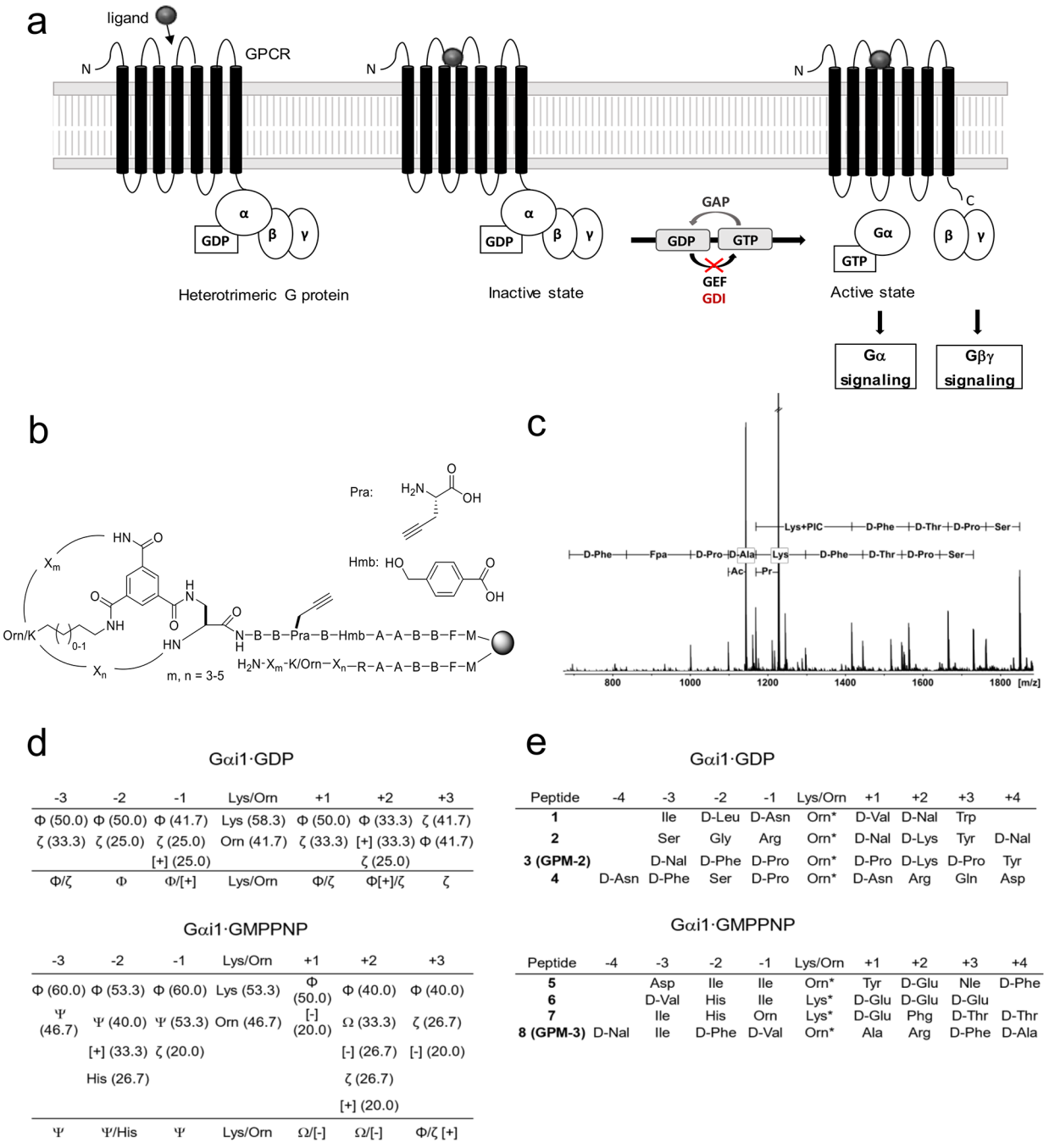


Figure 1. Development of a consensus sequence for G*αi* binding peptides based on the peptide library screening. (a) Schematic representation of G*α* protein signal transduction. (b) One-bead-two-compound (OBTC) library used in this study on TentaGel microbeads. The outer layer displays a unique bicyclic peptide, and the inner layer contains the corresponding linear peptide as an encoding tag. B: *β*-alanine; M: methionine; R: arginine; X: random amino acid residues; F: phenylalanine; Hmb, hydroxymethylbenzoyl; Pra, propargylglycine.[21−24] (c) Example of a representative PED-MALDI-MS spectrum of a hit sequence, i.e., Ser-D-Pro-D-Thr-D-Phe-Lys*-D-Ala-D-Pro-Fpa-D-Phe. (d) Amino acid preferences toward G*αi*1·GDP (top) and G*αi*1·GMPPNP (bottom) at the different positions are given relative to the central Lys/Orn. Relative frequencies of amino acid properties (≥20%) are given in symbols derived from Aasland et al.[25] as follows: Φ, hydrophobic (V, I, L, F, Fpa, Nal, Phg, W, Y, and M); Ω, aromatic (F, Fpa, Nal, Phg, W, and Y); Ψ, large aliphatic (V, I, L, and M); *ζ*, uncharged hydrophilic (N, Q, S, and T); [+], basic (H, K, Orn, and R); [-]: acidic amino acids (D and E). The derived trend is presented above. (e) Selected hit sequences from each screening experiment.

taken up by the cells. There are different mechanisms available by which peptides can penetrate the cell membrane;[14,19,34−36] when we tagged GPM-1-derived peptides with a CPP sequence, GPM-1c/d showed a GEM-like activity in cells.[7] This indicates that G*α* protein modulators can be obtained from combinatorial peptide library screening and with proper chemical alterations, such as the addition of unnatural amino acids,[37,38] can penetrate the cell and modulate intracellular cascades.[35,39] The potential of macrocyclic peptides modulating the G*α* protein activity is also evident from the selective G*αq* inhibitors FR900359 and YM-254890 (both depsipeptides), which have already found widespread utility in various pharmacological studies.[40−43] However, since the G*αq* subfamily is already well-targeted by the natural compounds,

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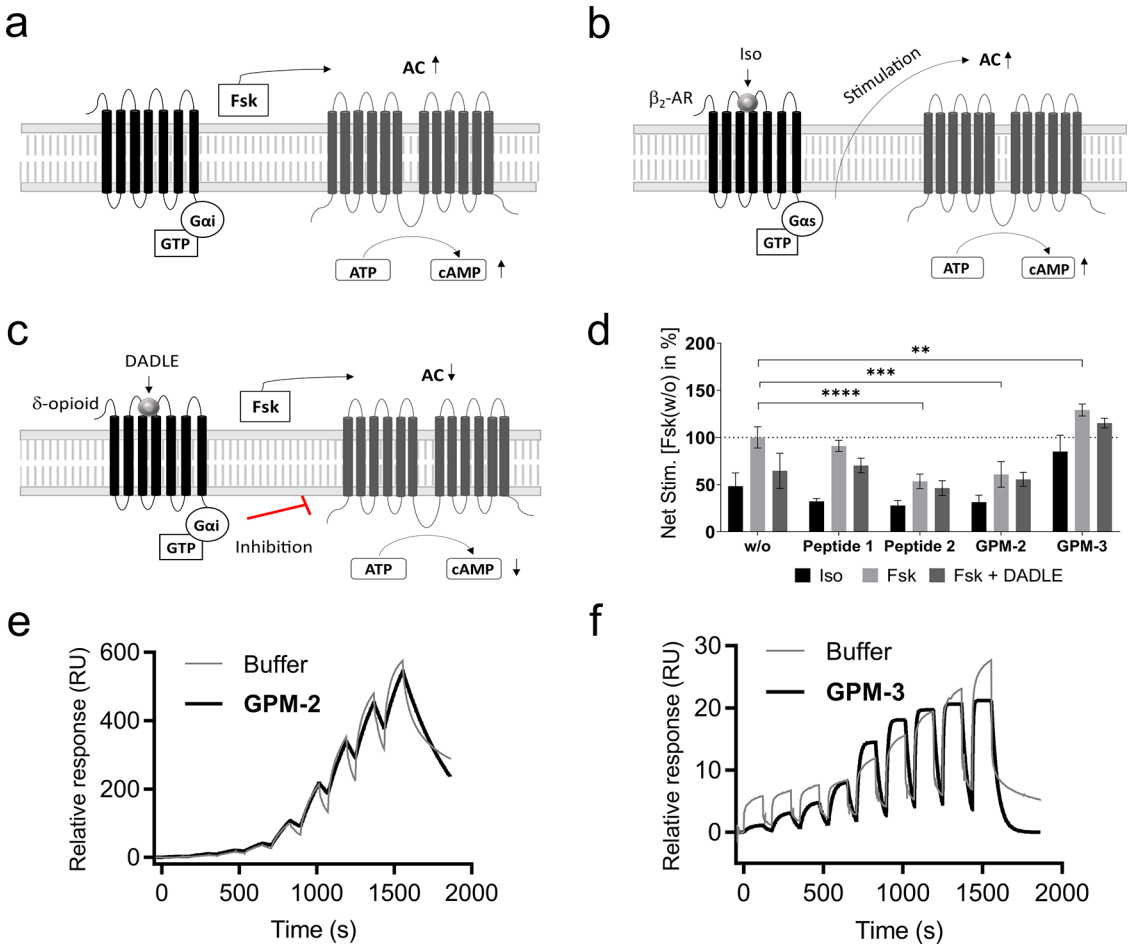

Figure 2. Preliminary studies of peptide selection (10 μM). (a–c) Illustration of membrane-based assays on the NG108−15 membrane preparations.[7,47] (a) Forskolin (Fsk) stimulates adenylyl cyclase (AC) and increases the cAMP level in a receptor-independent manner. (b) Upon isoproterenol (Iso) binding to the β_2-adrenergic receptor, Gαs signaling is activated, which in turn stimulates AC leading to an increased cAMP level. (c) Fsk + DADLE: Fsk stimulates cAMP production by direct activation of AC, whereas DADLE binds to the δ-opioid receptor, inhibits the direct AC activation by Fsk, and activates the Gαi signaling with a subsequent intracellular cAMP decrease. (d) Induced cAMP levels with or without ligand incubation, normalized to Fsk (no-peptide state) stimulation. Shown are percentage values of membranes incubated with the respective peptide in the presence of Iso, Fsk, and Fsk + DADLE. Error bars represent SD for $n = 3$. Statistical analysis was performed using the two-way ANOVA test, with $^{**}p < 0.001$, $^{***}p < 0.0003$, and $^{****}p < 0.0001$ for comparisons with the control (w/o). (e,f) SPR data of immobilized Gαi1•GDP with **GPM-2** (e) and Gαi1•GMPPNP with **GPM-3** (f), $n = 1$. (↑) Upregulation, (↓) downregulation.

we turned our attention to the Gαi subfamily, which has not yet been adequately addressed by either small molecules or peptides. In this study, we screened a bicyclic peptide library containing both proteinogenic and unnatural amino acids[9,18] against Gαi (in both active and inactive states). This led to the discovery of bicyclic peptides (e.g., **GPM-2** and **GPM-3**), which exhibit promising biological activities, e.g., on the production of second messenger cAMP. Further, computational analyses revealed the binding areas on the protein that could account for this biological activity. To the best of our knowledge, these represent the first peptidyl ligands with GAP activity toward the Gαi protein, in either the active and inactive state.

RESULTS AND DISCUSSION

Identification of State-Selective Gαi1 Binding Peptides by Screening of an OBTC Library. We previously demonstrated that linear peptidyl modulators of the Gα protein can be obtained by screening an OBOC combinatorial peptide library.[7] However, linear peptides are proteolytically labile and have poor cell permeability.[44] To overcome these limitations, we opted to screen a bicyclic peptide library, as the latter approach has recently led to the discovery of potent, cell-permeable, and metabolically stable bicyclic peptidyl inhibitors against the monomeric G protein K-Ras, namely, cyclorasin B3 and B4 (Figure 1b).[9] Given the structural similarity between monomeric G proteins and the GTPase domain of the Gα subunit of heterotrimeric G proteins, we hypothesized that Gα-binding peptides and modulators of Gαi might also be identified by screening a bicyclic peptide library. We chose a bicyclic OBTC peptide library previously reported by Lian et al.[18] and Upadhyaya et al.[9] In this library, each peptide ring contained three to five random residues, and each library bead displayed a unique bicyclic peptide (~50 pmol) on its surface and the corresponding linear peptide (~50 pmol) in its inner sphere. The peptide library was synthesized on 90 μm TentaGel beads (2.86×10^6 beads per gram) and has a theoretical diversity of 6.6×10^{13}. The peptide library was subjected to two rounds of screening against biotinylated (Btn-) Gαi in the inactive (GDP-bound) or active (GMPPNP-bound) state. In the first round, the bicyclic library (~5.4×10^5 beads) was incubated with Btn-Gαi protein and

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J. Med. Chem. 2023, 66, 12396−12406

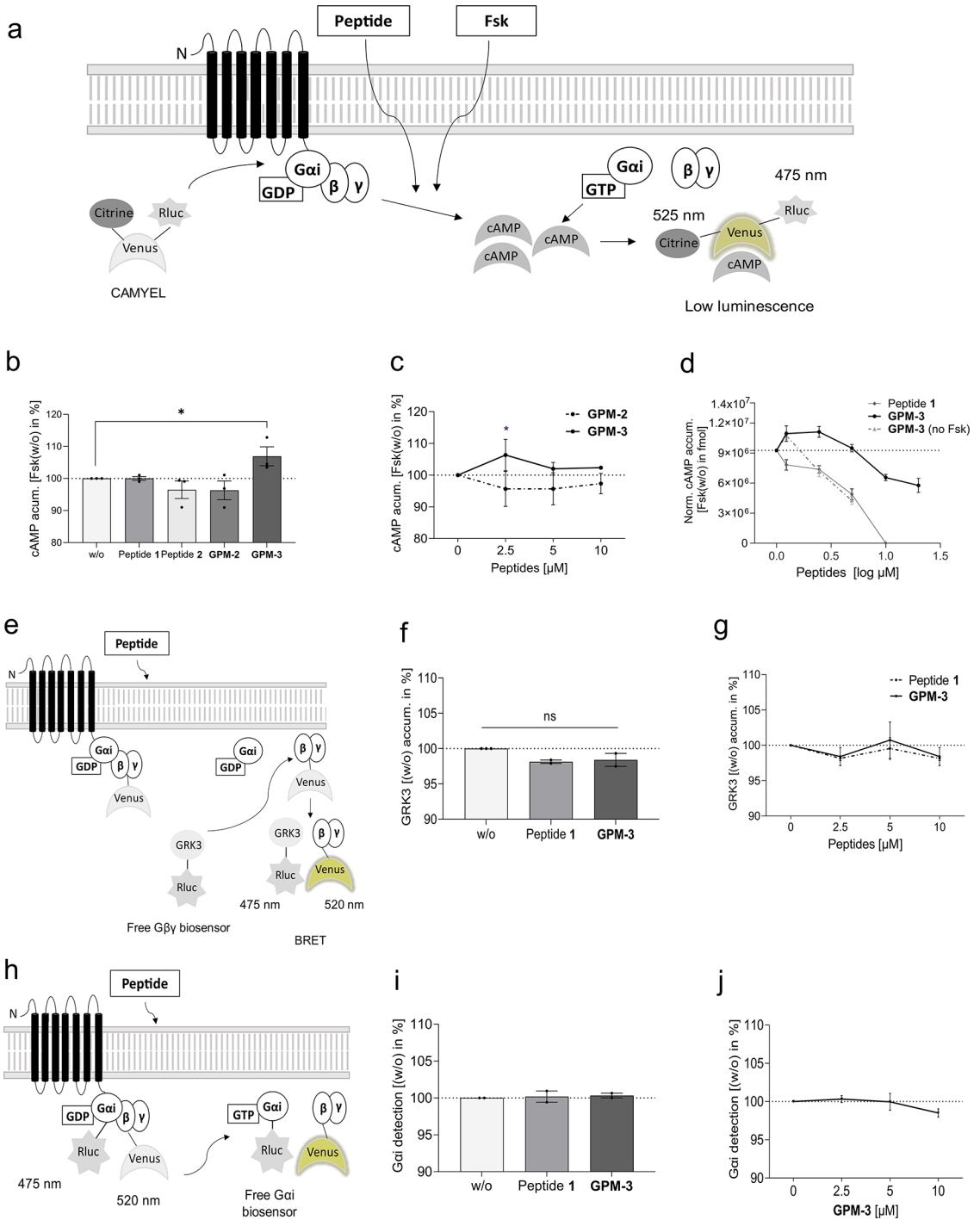

Figure 3. (a) Schematic representation of the BRET-based assays using a CAMYEL construct to detect the intracellular cAMP levels in live cells upon Fsk stimulation.[48,49] (b,c) The bar graph (b) represents the % cAMP production from CAMYEL-based BRET assays, where HeLa cells were incubated for 30 min with a 2.5 μM (or without) ligand. The resulting BRET signal was measured after cell stimulation with Fsk. A significant cAMP increase was observed for **GPM-3**. A concentration-dependent effect on the cAMP level is illustrated in panel c for **GPM-2**/**-3**. (d) End-point cellular studies (TR-FRET) for intracellular-cAMP level determination upon Fsk stimulation utilizing the LANCE cAMP kit. The quantification was facilitated by comparison to a cAMP calibration curve. (e,h) Schematic representation of the BRET-based assays using (e) a GRK3-Rluc or (h) Gαi-Rluc-fused construct that binds to free G$\beta\gamma$-GFP and induces BRET upon heterotrimer dissociation. (f,i) The bar graph (f) represents the % GRK3 (free G$\beta\gamma$ biosensor) production, whereas in (i), the % Gαi detection based on the presence of free G$\beta\gamma$ from the BRET assays (30 min incubation with a 2.5 μM (or w/o) ligand) is shown. No significant change was observed for **GPM-3**, assuming the Gαi heterotrimeric reassociation. (g,j) A concentration-dependent effect on the GRK3 level (g) or on Gαi detection (j) is illustrated for **GPM-3** and peptide **1** (neg. control). Error bars represent ±SEM for n = 2−3. Statistical analysis was performed using the one-way ANOVA test (Tukey's multiple comparison test), with $^*p < 0.05$ for comparisons with the control (w/o).

streptavidin-coated magnetic particles followed by isolation of the positive beads by magnetic sorting.[18] Next, the positive beads from above were incubated again with Btn-Gαi and a streptavidin-alkaline phosphatase (SA-AP) conjugate.[18,24]

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Subsequent incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)[24] resulted in a turquoise color on positive beads, which were manually isolated under a dissecting microscope. Thirty-five beads for Gαi·GDP and thirty-six for Gαi·GMPPNP were isolated, and their sequences were determined by the partial Edman degradation (PED)-mass spectrometry method (Figure 1c)[45,46] to give 12 and 15 complete, unambiguous sequences, respectively (Table S2 and S3). Four representative peptides against Gαi-inactive (peptides **1**−**4**) and four against Gαi-active (peptides **5**−**8**) were selected for resynthesis and testing (Figure 1e). Details on the establishment of the consensus sequence and peptide bioanalytical characterization are described in the Supporting Information (Figures S1−S4 and Table S4).

Validation of Library Hits by Membrane-Based Assays and SPR. To identify bona fide Gα modulators, peptides **1**−**8** were initially tested in a cell-free ELISA for their effect on the function of AC.[7,47] As depicted in Figure 2, all assays were performed using a membrane preparation from NG108−15 hybrid cells stably expressing human β_2-AR, where the final readout is taken into consideration. Three different systems were tested: first, a receptor-independent and then a β_2-AR-bound (specific for Gαs) system, where NG108−15 membrane preparations were stimulated with forskolin (Fsk, Figure 2a) and isoproterenol (Iso, Figure 2b), respectively, stimulating the subsequent cAMP production. Third, a δ-opioid-bound (specific for Gαi) approach was used, where DADLE binds to the receptor and prevents the activation of AC (stimulated by Fsk, Figure 2c). The studies indicated that among all compounds (Figure S5), when compared to the net Fsk stimulation, peptide **3** (referred to as **GPM-2** hereafter, 10 μM) causes a ∼40% decrease of the cAMP production (or activating Gαi signaling), whereas peptide **8** (**GPM-3**) increased the cAMP level (Fsk and Fsk + DADLE stimulation) by ∼30%, indicating an opposite effect on the Gαi signaling with a possible inhibitory activity (Figure 2d). The peptide specificity against Gαi signaling was tested upon Iso stimulation, where no notable change was distinguished. Peptide **1** exhibited no significant effect on Gαi signaling and was used as a negative control for further studies.

We next tested four of the eight peptides (**1** as a negative control, **2**, **GPM-2**, and **GPM-3**) for binding to Gαi by surface plasmon resonance (SPR) analysis. Gαi•GDP/GMPPNP was immobilized onto the surface of a sensor chip (via amine coupling, Figure S6a−f and Table S5), and varying concentrations of the peptides were flown over the surface. **GPM-2** and peptide **2** bound to Gαi•GDP with K_D values of 5.0 ± 0.34 (Figure 2e) and 24.7 ± 0.95 μM (Figure S6b and Table S5), respectively. In the case of **GPM-2**, the observed binding affinity lies within the range of previously reported dissociation factors of the used screening library (1−10 μM).[9,18,38] **GPM-3** bound to Gαi•GMPPNP with a K_D value of 0.71 ± 0.37 μM (Figure 2f); as expected, it bound to Gαi•GDP with a much lower affinity (K_D = 25.5 μM, Figure S6c and Table S5). Also as expected, the negative control peptide **1** did not bind Gαi•GDP (Figure S6a). Binding assays were also performed in the reverse direction, by immobilizing the peptides on the SPR surface and flowing protein solutions over the surface. No binding was observed, presumably because the immobilization procedure modified the peptide structure, thereby interfering with protein binding (Figure S6g−i). To our knowledge, **GPM-3** represents the first potent, selective bicyclic peptidyl ligand with a sublow-μM binding affinity for the GMPPNP-bound Gαi protein.[9,23,26−28]

GPM-3 Induces Gαi-GTP Hydrolysis Attaining a GAP Function. The cell-free studies suggest that **GPM-3** may possess GAP activity. To test this notion, we subsequently assayed the selected bicyclic peptides in HeLa cells. To assess the AC activation, we performed a series of receptor-independent BRET studies (bioluminescence resonance energy transfer) by utilizing a well-established system (Figure 3a), which monitors the intracellular cAMP levels in real time and in live cells with a CAMYEL (cAMP sensor using YFP-Epac-RLuc) construct.[48,49] Initially, the cells were incubated with varying peptide concentrations (0−10 μM, Figure S7a), and cAMP production was measured upon Fsk stimulation. These experiments revealed that for all three peptides, a 2.5 μM ligand is optimal for exerting an effect on AC (Figure 3b,c). **GPM-3** was most active in this assay and increased the cAMP levels by ∼10%, which is in agreement with the results from the membrane-based assays. Peptides **GPM-2** and **2** caused a decrease of the cAMP level, although with lower potencies compared with the data from the membrane assays. Again, the negative control peptide **1** had no significant effect. This consistency in elevating cAMP levels, in combination with the fact that **GPM-3** binds preferentially to the active state of Gαi, suggests that **GPM-3** is a potential GAP modulator. To assess the cellular selectivity of **GPM-3** for Gαi, we conducted the same BRET approach with cells expressing β_2-AR (which is Gαs-specific) and stimulated the cells with Iso, in a manner similar to that of the membrane assays. The results indicated the subfamily specificity of **GPM-2**/**-3** since no significant change in the cAMP levels was observed (Figure S7b,c). The cellular activity of the bicyclic peptides indicates that they are permeable to the cell membrane. Inspection of their structures revealed the presence of both aromatic hydrophobic and positively charged amino acids, which are the key features of CPPs.[14] It should be noted that the modest activity of peptides **2** and **GPM-2** could result from their lower cell permeability compared to **GPM-3** and that further optimization of the bicyclic peptides, e.g., conjugation to a highly active CPP, may further enhance the cellular activity of these peptides. None of the bicyclic peptides showed significant cytotoxicity up to an 80 μM concentration (by the MTT assay[7]). **GPM-3** showed excellent stability in human plasma, with no significant degradation after 180 min (Figure S8).[50] These attributes led us to select **GPM-3** as a lead compound for further investigation.

Quantitative Determination of the GTPase Activity of GPM-3. Next, we aimed to get deeper insight into the induced GTP hydrolysis function of **GPM-3** with quantitative assays. First, we determined the end-point intracellular cAMP levels with a TR-FRET (time-resolved fluorescence resonance energy transfer) assay.[51] HeLa cells were incubated with different **GPM-3** concentrations, and cAMP production was stimulated with Fsk (or without). The LANCE Ultra cAMP kit was utilized to quantify the cAMP levels (Figure 3d, compared with a cAMP standard curve). The outcome was in accordance with our previous results since a low concentration of **GPM-3** (2.5 μM, Fsk-stimulated) induced an elevated cAMP level first, which is then reduced upon increasing peptide concentrations, revealing an IC_{50} value of 6.92 μM.

GPM-3 Hints at a Possible Heterotrimer Reassociation. To assess the role of **GPM-3** as a GAP for Gαi, we performed additional BRET assays and monitored the levels of

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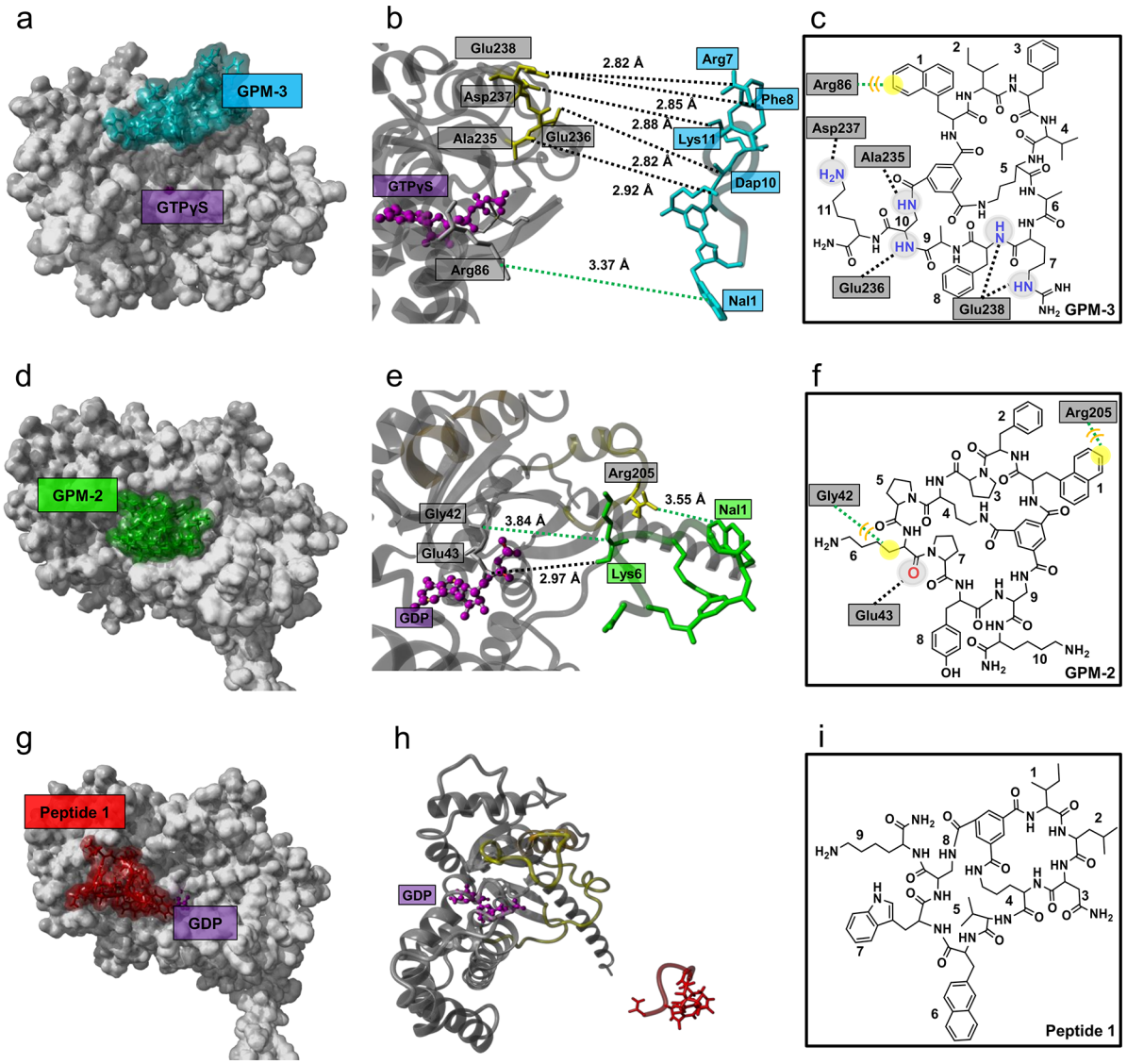

Figure 4. Overview illustration of protein−ligand complexes formed between bicyclic peptidic modulators and Gαi1·GTPγS/GDP. In a−g, the binding sites of **GPM-3** (cyan), **GPM-2** (green), and peptide **1** (negative control, red) are shown on the van der Waals surface (gray) of the respective Gαi1 structure. Structures (b, e, and h) illustrate the interactions between the side chains of the interacting residues on the receptor with the respective sides of the bicyclic ligands. The respective bound nucleotide (magenta) and the interacting residues (color coding as shown above) are labeled. Hydrogen bonding and hydrophobic interactions are represented by black and green dotted lines, respectively. At the same time, switch regions are highlighted in yellow, and the α3-helix is displayed in orange. In c, f, and i, the chemical structure of each bicyclic peptide and their atom interactions (oxygen in red, nitrogen in blue) with surrounding protein residues at the binding sites are depicted in details. PDB IDs: Gαi1·GTPγS (1GIA[54]) and the homology model of Gαi1·GDP[7] (PDB IDs 1Y3A,[29] 5JS8,[60] and 3UMS[61]).

free GRK3 (C-terminus of G protein-coupled receptor kinase-3, also GRK3ct) and the release of the Gβγ dimer (Figure 3e), or the levels of the free monomeric Gαi subunit (Figure 3f). GRK3ct associates with free Gβγ and therefore acts as a G protein dissociation indicator.[52] This is a Gβγ activation assay, where upon incubation with the respective ligand, the cells bearing the Rluc-fused GRK3ct or Rluc-Gαi should lead to heterotrimer dissociation and subsequent release of the Gβγ dimer (and interaction with the GRK3) or the Gαi subunit inducing eventually the BRET signal. The data indicate that **GPM-3** consistently (albeit only slightly) reduced the levels of both the free Gβγ dimer (Figure 3f,g) and monomeric Gαi (Figure 3h−j), suggesting that the **GPM-3** may evoke the heterotrimer reassociation. Also, release of the Gβγ dimer could also support the activation of the Akt/ERK pathway.[53] Immunoblots of HeLa cell lysates incubated with peptides **1**, **2**, **GPM-2**, and **GPM-3** for determining the phosphorylated (p-) and total (t-) Akt and ERK showed a slight effect for **GPM-2** and no effect for **GPM-3** on the Akt/ERK pathway (Figure S9), supporting our suggestion of heterotrimer reformation in the latter case.

Identification of Novel Binding Sites by *In Silico* Analyses. No structural information is currently available for the binding of macrocyclic peptidyl ligands to the Gαi protein.[26−28] We therefore turned to computational modeling to gain some insight into the binding of peptides **1**, **2**, and **GPM-2** to Gαi·GDP by using a previously established homology model[2,7] and docking **GPM-3** to the existing structure of Gαi1·GTPγS (PDB 1GIA,[54] Figures S10−17 and Tables S6 and S7). Based on the results of 100 ns

https://doi.org/10.1021/acs.jmedchem.3c00873
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molecular dynamics (MD) simulations, we observed that **GPM-3** and **GPM-2** form stable complexes with Gαi via distinct stabilizing interactions (Figure 4a−c and d−f, respectively). Conversely, peptide **1** dissociates from its binding site over the course of the simulation with a conformational change of 1.64 Å (Figure 4g−i), while peptide **2** forms an unstable complex with the inactive Gαi protein (Supporting Information). **GPM-3** binds between the αA (α-helical domain, α-HD) and switch III region (SWIII, GTPase domain) and thus interacts with both domains of Gαi1·GTPγS (Figures S12 and S13). This binding site has not previously been occupied by any of the other peptidic GEM modulators.[7,32] **GPM-3** persistently interacts with α-HD through hydrophobic interactions between the aromatic side chain of Nal1 and residue Arg86 in αA (Figure 4a−c). Additional interactions are mediated by hydrogen bonds between the following pairs of residues: Arg7-Glu238, Phe8-Glu238, Dap10-Ala235 and Dap10-Glu236, and Lys11-Asp237, which occur at frequencies of >80% (Figure 4b,c and Video S2). The critical role of Lys11 explains why immobilization of GPM-3 to the sensor chip prevented Gαi binding in our SPR experiments. Residues Ala235, Glu236, and Asp237 are closely associated with the GTP hydrolysis mechanism[54−57] and interaction with the GAP protein RGS4.[7,55] The observation that **GPM-3** interacts with these three residues is consistent with **GPM-3** acting as a GAP. Three critical residues, Arg_{cat}178, Thr181, and Gln_{cat}204, are known to be key players during GTP hydrolysis.[56,58,59] Herein, Arg_{cat}178 forms a salt bridge with Glu43 (P-loop) and acts as a "seatbelt", stabilizing the nucleotide at the binding site.[29] This bridge persists throughout the simulation trajectory, indicating that the transition state is indeed conserved (Figure S15 and Video S3).From another perspective, the heterotrimer formation was examined via simulations based on the Gβγ behavior. Commonly, the "attraction" between the SWII and SWIII regions is enhanced by the interactions of Glu236 and Asp237 (SWIII) with Arg205 (SWII).[57] Surprisingly, **GPM-3** potentiates the interactions between Arg205-Glu236 and Arg205-Asp237 by stabilizing the orientations of the side chains of Glu236 and Asp237 converging them in SWII (Figure S16 and Video S2). Another interesting property of **GPM-3** is the enfeebling of the Gly203-Arg208 interaction within the G-R-E motif (Gly203-Arg208-Glu245).[57] The formation of the G-R-E triad usually favors the dissociation of Gβγ from the heterotrimer by reducing the strength of the polar network formed between Lys209/210 (SWII) and the Gβγ dimer. The weakening of the interaction of Gly203-Arg208 leads to triad perturbation and subsequent suppression of the Gβγ release (Figure S16). This triad state may be an indication of the protein transition from the active to inactive state. The persistence of the salt bridge (stable transition state) in the simulation trajectory and the prevention of triad formation (heterotrimer reassociation) strengthen our assertion of **GPM-3** as a GAP for the Gαi protein. In addition to that, **GPM-3** has also been analyzed against the heterotrimeric Gαi1β1γ2·GDP (PDB 1GP2[62]) complex (Figure S17). Despite the peptide binding on the heterotrimer, there is a minor shift after 100 ns that is observed. Although GPM-3 mainly interacts with the SWIII region, the interactions are not as frequent as in the case of the Gαi1·GTPγS complex (Figure 4a−c). The above results provide unique information about the computationally assisted structural depiction of the bicyclic peptide interaction with the active state of the Gαi protein. Unlike **GPM-3**, **GPM-2** binds between both domains but is "localized" in front of the nucleotide binding pocket, another undescribed binding site (Figure 4d and Figure S13). The peptide participates in multiple hydrogen bonds (50%) through Lys6, as well as in stable hydrophobic interactions (>80%) via both Lys6 and Nal1 at this binding site. Since Lys6 contributes to both types of interactions, it appears to be the essential residue for complex stability. In particular, this residue interacts through its Cβ atom with the Cα of Gly42, which is entangled in the catalytic motif in the P-loop (Figure 4e,f), whereas Nal1 forms a hydrophobic bond with Arg205 and stabilizes the complex. Additionally, it was observed that different residues within the peptide interact with Glu43 leading to interruption of the salt bridge Arg178-Glu43. Thus, the peptide may enable GDP exchange by partially interfering with the salt bridge formation.[29] To evaluate the topological behavior of **GPM-2** for the nucleotide exchange, we also conducted a 100 ns MD simulation of the Gαi "empty pocket" state. The results denoted that the peptide slowly lost its interactions with the surrounding protein residues, dissociated from the binding site already after 10 ns, and continued over the entire simulation trajectory (Video S4). Here, the peptide did not block the nucleotide binding site. These data corroborate the binding and cellular studies where **GPM-2** binds to the protein and moderately activates the Gαi signaling through nucleotide exchange. Moreover, the instability and dynamic behavior of peptides **1** and **2** in their respective complexes during simulation proved the findings of the experimental studies (detailed description provided in the Supporting Information).

Overall, based on our experimental and computational observations, we hypothesize that the new binding sites may be suitable regions for the development of peptidic modulators with novel biological activity.

CONCLUSIONS

The present study provides a novel series of moderately potent, selective, cell-permeable, and metabolically stable bicyclic peptidyl Gαi modulators. One of the peptides, **GPM-3**, is biologically active in cellular assays and has an AC stimulating effect on the active state of the Gαi protein but no significant effect on the Gαs subunit. On the other hand, peptides **2** and **GPM-2** target the Gαi·GDP state and exhibit a partial AC inhibitory effect, with the latter also exhibiting the ability to promote GDP exchange. Computational studies provided key insights into the binding of the bicyclic peptides to the Gαi protein and the structural basis for **GPM-3** acting as a GAP for Gαi. We anticipate that **GPM-3** may be further improved through structural optimization, e.g., by conjugation with a CPP to enhance its cell penetration properties. Due to the significant biomedical relevance of G proteins[2,63,64] and the lack of potent and selective Gαi/s modulators, the strategy described can lead to promising compounds for further drug development and pharmacokinetic investigations.

EXPERIMENTAL SECTION

One-Bead-Two-Compound (OBTC) Library Screening. Screening experiments with the Gαi protein were performed using a bicyclic peptide library[9,18] according to Lian et al.[18] and Qian et al.[36] Therefore, the bicyclic peptide library was subjected to two rounds of screening against the biotinylated Gαi protein. During the first round, 180 mg (approach with Btn-Gαi·GDP: 272 mg) of the bicyclic library was incubated with biotinylated 0.5 μM Gαi [inactive GDP-bound or

https://doi.org/10.1021/acs.jmedchem.3c00873
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the active state of GMPPNP-bound (guanosine-5′-[(β,γ)-imido]-triphosphate)] and streptavidin-coated magnetic particles. The resulting magnetic beads were isolated from the library by magnetic sorting, during which the positive beads were attracted to the wall, while the negative beads settled at the bottom of the container. The positive beads were washed, incubated again with the biotinylated G protein (0.2−0.5 μM), and subjected to a second round of screening. This screening approach was based on an on-bead enzyme-linked assay and a streptavidin-alkaline phosphatase (SA-AP) conjugate, in which binding of the G protein to a bead recruited SA-AP to the bead surface. Upon the addition of BCIP (5-bromo-4-chloro-3-indolyl phosphate), SA-AP produced a blue precipitate on the protein-bound bead. The intensely colored beads were manually isolated with a micropipette and subjected to PED-MALDI-MS analysis for sequencing the resin-bound peptides according to Sweeney[46] and Thakkar and Wavreille.[45] The identified hit sequences are listed in Tables S2 and S3 along with the establishment of the consensus sequence.

Peptide Synthesis and Purification. Solid-phase peptide synthesis according to Lian et al.[23] was performed on Rink Amide MBHA resin (0.53 mmol·g^{-1}) with HATU (4 equiv) as a coupling reagent and NMM (8 equiv) as a base. For the coupling of phenylglycine, COMU/TMP was used as described previously.[65] In order to reduce the resin loading to 0.25−0.33 mmol·g^{-1}, Fmoc-Lys(Boc)−OH (0.6 equiv) was coupled first followed by acetylation (1 equiv of acetic anhydride, 2 equiv of *N*-methylimidazole in DMF, 30 min) to block the remaining free amino groups. Subsequently, Fmoc-Dap(Aloc)−OH and the respective amino acid sequence (Fmoc-protected amino acids) as well as trimesic acid were coupled as reported earlier.[23] The Aloc group was cleaved by $Pd(PPh_3)_4$ (0.5 equiv) and phenylsilane (10 equiv) in DCM, and then, cyclization with PyBOP (10 equiv) and HOBt (10 equiv) was performed on the resin. The cleavage of the side-chain protecting groups together with the respective peptides from the resin was performed with reagent K as described before.[21] The crude peptides were purified by semipreparative reversed-phase HPLC using a Shimadzu LC-8A instrument, and the subsequent purity of the peptides (>95%) was confirmed by analytical RP-HPLC from a Shimadzu LC-20AD system. The collected fractions were combined, freeze-dried, and stored at −20 °C. Detailed information on the purification and analysis of individual peptides can be found in Table S4.

Live-Cell Biological Activity Assay. HeLa cells from American Type Culture Collection (ATCC) were cultured and maintained accordingly[48] in DMEM supplemented with 10% FBS. For the intracellular cAMP and Gβγ measurement assay, a BRET (bioluminescence resonance energy transfer)-based experimental setup was used.[49] The cAMP-based sensor was a generous gift from Dr. Tracy Handel and Dr. Irina Kufareva (University of California, San Diego). On day one, cells were plated (400,000 cells per well). On the second day, these cells were transfected with a total of 2000 ng of total DNA with Gαi (C351I)/β_2-AR (1000 ng) or pcDNA and modified CAMYEL (1000 ng). For the Gβγ release measurements, the cells were transfected with Venus-fused Gβ (500 ng), Gγ (500 ng), and Rluc8-fused Gαi (500 ng) plasmids.[51] Transfection was performed according to the manufacturer's protocol for the TransIT-X2 transfection reagent (Mirius, USA). On day three, cells were washed and replated on a 96-well plate (white, clear bottom) supplemented with DMEM with 10% FBS at a density of 40,000 cells per well. On day four, the culture medium was removed, and 70 μL of assay buffer (0.027 mM glucose, 250 μL of BSA, and 50 mL of PBS pH 8.0) was added. Peptide solutions (final concentrations of 10, 5, and 2.5 μM, diluted in assay buffer) were added (10 μL) to the cells and incubated for 30 min at RT. The luminescence scan was performed after the peptide treatment on the plate reader (Tecan, Spark M20, Switzerland). The initial cellular luminescence was measured for 7 min after the addition of 10 μL of a mixture of 100 μM IBMX (for cAMP detection) and 10 μM CTZ (in assay buffer). With regard to the CAMYEL release, the BRET was measured again for 15 min after the addition of 10 μL of 5 mM Fsk or 1 mM isoproterenol. The final BRET ratio was calculated from the excitation and emission scan. The bar graphs were generated from normalizing the area under the curve (AUC) values of the no-peptide and peptide-treated conditions. The assay was validated in triplicate, and the final outcome was plotted.

For the GRK3 measurement, a different but similar BRET approach was used.[51,66] HeLa cells from American Type Culture Collection (ATCC) were cultured and maintained accordingly[66] in DMEM supplemented with 10% FBS. On day one, cells were plated (400K cells per well). On the second day, these cells were transfected with a total of 2,000 ng of total DNA with GRK3-Rluc (50 ng) or pcDNA, Gαi (500 ng), and Gβ- (500 ng) and Gγ-fused (500 ng) GFP in a 1:1:1:1 ratio. Transfection was performed according to the manufacturer's protocol for the TransIT-X2 transfection reagent (Mirius, USA). On day three, cells were washed and replated on a 96-well plate (white, clear bottom) supplemented with DMEM with 10% FBS at a density of 40,000 cells per well. On day four, the cell medium was removed, and 70 μL of assay buffer (0.027 mM glucose, 250 μL of BSA, and 50 mL of PBS pH 8.0) was added. Peptide solutions (final concentrations of 10, 5, and 2.5 μM, diluted in assay buffer) were added (10 μL) to the cells and incubated for 30 min at RT. The BRET luminescence was measured after the addition of 10 μL of 2 mM CTZ (coelentrazine) in assay buffer, utilizing a plate reader (Tecan, Spark M20, Switzerland).

For the Gαi detection, the same HeLa cell lines were used (400,000 cells per well) with the following modifications: On the second day, these cells were transfected with a total of 1,500 ng of total DNA with GRK3-Rluc (50 ng) or pcDNA, Gα-Rluc (500 ng), and Gβ- (500 ng) and Gγ-fused (500 ng) GFP, and the transfection was facilitated via the TransIT-X2 reagent. Cell plating and peptide preparation were handled in the same way as described above, and the BRET signal was recorded with the plate reader after the addition of 10 μL of 2 mM CTZ in assay buffer. The bar graphs were generated from normalizing the area under the curve (AUC) values of the no-peptide and peptide-treated conditions. The assay was validated in three independent measurements (n = 3, each one performed in triplicate), and the final result was plotted via GraphPad Prism 8.0.

***In Silico* Studies.** Each bicyclic peptide was generated manually on YASARA (structure, version 21.8.27)[67] based on the spatial organization of the amino acid residues (Figure S6). The dynamic behavior and stability of the elucidated bicyclic peptides were monitored after 50 ns MD simulations. The derived peptide structures were then used for docking studies and binding site investigations. For these purposes, a homology model (HM) of Gαi1·GDP, which was generated as previously[7] from PDBs 1Y3A,[29] 3UMS,[61] and 5JS8,[60] was used as the target protein for the bicyclic peptides **1**, **2**, and **GPM-2** (binders of the inactive protein state), whereas the Gαi1·GTPγS (PDB 1GIA[54]) and Gαi1β1γ2·GDP (PDB 1GP2[62]) structures were tested for **GPM-3**. The peptides were first docked to the hydrophobic cleft between the SWII/α3-helix (focused docking approach in YASARA). Due to no stable binding, further studies targeting potential alternative binding surfaces were conducted and additionally supported by SeeSAR (BioSolveIT GmbH, Version 11). Blind and ensemble docking studies were performed to determine the optimal peptide binding poses. The most suitable ones were determined, and the protein−ligand complexes were further subjected to 500 ps refinement simulations with the YAMBER[68] force field to increase the accuracy and quality of the selected complex. The resulting protein−ligand complex with the lowest energy and the highest structural quality was then subjected to 100 ns MD simulations to monitor the stability of the complexes formed and to investigate the effects of the peptides on the protein. A detailed description of the computational methodology is provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.3c00873.

Standard experimental procedures and instrumentation used (Table S1); text and supporting figures about protein and peptide characterization (Figures S1−S4

https://doi.org/10.1021/acs.jmedchem.3c00873
J. Med. Chem. 2023, 66, 12396−12406

and Tables S2−S4), additional data for membrane-based (Figure S5), binding (SPR, Figure S6 and Table S5), plasma stability (Figure S8), and biological (Figures S6, S9, and S10) studies; computational analysis and data sets of protein−peptide interactions (Figures S10−S17 and Tables S6 and S7) (PDF)
(Video S1) MD simulation of the GPM-3−Gαi1·GTPγS complex (MP4)
(Video S2) Interactions of Glu236 and Asp237 (SWIII) with Arg205 (SWII) and perturbation of the G-R-E triad (MP4)
(Video S3) Salt bridge between Arg178 and Glu43 (MP4)
(Video S4) Empty pocket simulation of the complex GPM-2−Gαi1 (MP4)
(Video S5) Simulation of the peptide 1−Gαi1 (empty pocket) complex (MP4)
Molecular formula strings file (CSV)
PDB file for 1GIA (PDB)
PDB file for 1GP2 (PDB)
PDB file for Gai1_HM (PDB)
PDB file for 1Y3A (PDB)
PDB file for 3UMS (PDB)
PDB file for 5JS8 (PDB)

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Author Contributions

D.I. conceived and designed the research project with the support of A.P., D.P., and P.G.; A.P. performed the expression and purification of the Gαi protein; B.N. performed the library screening and PED-MALDI-MS analysis with the support of A.K., C.A.R., D.P., and D.I.; the peptide synthesis, purification, and analytical characterization were conducted by B.N. and A.P.; H.A., B.N., A.P., and D.I. performed and analyzed the membrane-based assays; A.P. performed and analyzed the SPR studies; A.P., S.R., and P.G. designed, performed, and analyzed the cellular biological activity studies; F.A.B. carried out and analyzed the computational studies supported by A.P. and D.I.; all authors discussed the results and contributed to the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support by the University of Bonn (to D.I.), the Deutsche Forschungsgemeinschaft (DFG) within FOR 2372 and IM 97/14-1 (to D.I.), the Heinrich Hertz-Stiftung (to A.P.), the Bonner Universitätsstiftung (to B.N.), and National Institute of Health (NIH) R01 Awards CA100768, CA238042, and AI141630 (to P.G.) and R35 GM122459 (to D.P.) is gratefully acknowledged. We want to express our gratitude to T. Kühl, N. Bardysh, and S. Linden (all from the University of Bonn) for technical support and A. A. Paul George (BioSolveIT GmbH) for the scientific discussion and BioSolveIT for providing access to SeeSAR software, M. Geyer (University Hospital of Bonn) for support and access to the Biacore instrument, and Solvay GmbH for the friendly supply of TFA.

ABBREVIATIONS

AC, adenylyl cyclase; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; CPP, cell-penetrating peptide; GAP, GTPase-activating proteins; GDI, guanine-nucleotide dissociation inhibitor; GEF, guanine-nucleotide exchange factor; GEM, guanine-nucleotide exchange modulator; GPCR, G protein-coupled receptor; Fsk, Forskolin; Iso, isoproterenol; MD, molecular dynamics; SPR, surface plasmon resonance; SW, switch regions; TR-FRET, time-resolved fluorescence resonance energy transfer

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Publications

Manuscripts

1. B. Nubbemeyer, **A. Pepanian**, A. A. Paul George, and D. Imhof (2021) Strategies towards targeting Galpha(i/s) proteins: Scanning of protein-protein interaction sites to overcome inaccessibility. *ChemMedChem*., 16, 1-21. DOI: 10.1002/cmdc.202100039.
2. B. Nubbemeyer, A. A. Paul George, T. Kühl, **A. Pepanian**, M. S. Beck, R. Maghraby, M. A. Shetab Boushehri, M. Muehlhaupt, E. M. Pfeil, S. K. Annala, H. Ammer, D. Imhof, and D. Pei (2022) Targeting Gαi/s Proteins with Peptidyl Nucleotide Exchange Modulators. *ACS Chem. Biol*. 17, 463–473. DOI: 10.1021/acschembio.1c00929.
3. M.-T. Hopp, A. A. Paul George, A. Ramoji, **A. Pepanian**, M. S. Detzel, U. Neugebauer, and D. Imhof (2022) A model peptide reveals insights into the interaction of human hemopexin with heme. *Int. J. Pept. Res. Ther*. 28, 129. DOI: 10.1007/s10989-022-10441-x.
4. **A. Pepanian***, P. Sommerfeld*, R. Kasprzyk*, T. Kühl, F. A. Binbay, C. Hauser, R. Löser, R. Wodtke, M. Bednarczyk, M. Chromiński, J. Kowalska, J. Jemielity, D. Imhof, and M. Pietsch (2022) Fluorescence Anisotropy Assay with Guanine Nucleotides Provides Access to Functional Analysis of Gαi1 Proteins. *Anal. Chem*. 94, 14410-14418. DOI: 10.1021/acs.analchem.2c03176.
5. **A. Pepanian**, F. A. Binbay, S. Roy, B. Nubbemeyer, A. Koley, C. A. Rhodes, H. Ammer, D. Pei, P. Ghosh, and D. Imhof. (2023) Bicyclic peptide library screening for the identification of Gαi protein modulators. *J. Med. Chem.* (accepted).
6. **A. Pepanian**, P. Sommerfeld, F. A. Binbay, D. Fischer, M. Pietsch, and D. Imhof. (2023) In-depth characterization of Gαs protein activity by probing different guanine nucleotides. *Protein Sci.* (in review).

Oral Presentations

1. Oral presentation (invited) in the frame of the iStart Project of the International Office of the University of Bonn: “International Alumni members as role models”, Bonn, Germany (May 12, 2021).
2. Oral presentation (invited) entitled “Modulation of Gαi1/s proteins with molecules targeting the guanine nucleotide binding pocket”, Gordon Research Conference: Phosphorylation and G-Protein Mediated Signaling Networks, Southbridge, USA (June 12 – 17, 2022).
3. Oral presentation (volunteered) entitled “Investigation of potent Gα protein modulators”, BIGS DrugS kick off meeting, Bonn, Germany (July 11, 2022).

Scientific Posters

1. “Targeting of Gαi/s Protein by Peptidic Guanine Nucleotide Exchange Modulators.” Advances in Chemical Biology, virtual/Frankfurt am Main, Germany (January 28, 2021).
2. “Targeting Gαi/s Proteins with peptidyl nucleotide exchange modulators.” European Peptide Symposium (August 2022), Sitges, Spain (August 28 – September 2, 2022).
3. “Modulation of Gαi1/s proteins with molecules targeting the guanine nucleotide binding pocket.” Gordon Research Conference: Phosphorylation and G-Protein Mediated Signaling Networks, Southbridge, USA (June 12 – 17, 2022).
4. “Nucleotide exchange modulation of Gαi/s proteins with linear and macrocyclic peptides.” German Peptide Symposium, Jena, Germany (August 28 – 30, 2023).