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Abstract

G proteins represent intracellular switches that transduce signals obtained from G protein-coupled receptors. The structurally related macrocyclic depsipeptides FR900359 (FR) and YM-254890 (YM) are potent, selective inhibitors of the Goq protein family. We recently discovered that radiolabeled FR and YM display strongly divergent residence times, which translates into significantly longer antiasthmatic effects of FR. The present study is aimed at investigating the molecular basis for this observed disparity. Based on docking studies, we mutated amino acid residues of the Goq protein predicted to interact with FR or YM, and recombinantly expressed the mutated Goq proteins in cells in which the native Goq proteins had been knocked out by CRISPR-Cas9. Both radioligands showed similar association kinetics, and their binding followed a conformational selection mechanism, which was rationalized by molecular dynamics simulation studies. Several mutations of amino acid residues near the putative binding site of the “lipophilic anchors” of FR, especially those predicted to interact with the isopropyl group present in FR but not in YM, led to dramatically accelerated dissociation kinetics. Our data indicate that the long residence time of FR depends on lipophilic interactions within its binding site. The observed structure-kinetic relationships point to a complex binding mechanism of FR, which likely involves snap-lock- or dowel-like conformational changes of either (or both) ligand and protein. These experimental data will be useful for the design of compounds with a desired residence time, a parameter that has now been recognized to be of utmost importance in drug development.
List of Abbreviations

BSA, bovine serum albumin; CDK8/CycC, cyclin dependent kinase 8/Cyclin C; cpm, counts per minute; DMEM, Dulbecco’s modified Eagle medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FR, FR900359; GDP, guanosine diphosphate; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; HA, hemagglutinin; HBSS, Hank’s balanced salt solution; HEK, human embryonic kidney; HRP, horseradish peroxidase; IP₃, inositol trisphosphate; KO, knockout; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline + 0.1% Tween 20; PCR, polymerase chain reaction; PLC-β, phospholipase C-β; RasD, Ras-like domain; SDS, sodium dodecyl sulfate; VSV-G, vesicular stomatitis virus G; wt, wild-type; YM, YM-254890; αH, α-helical domain

1. Introduction

Heterotrimeric guanine nucleotide binding proteins, G proteins, are crucial switches that transmit extracellular signals received by G protein-coupled receptors (GPCRs) across cellular membranes [1,2]. In the basal, inactive state, the GDP-bound Gα subunit is associated with the βγ-dimer [3]. Upon GPCR activation, the receptor couples to the G protein and triggers the exchange of GDP for GTP [4]. This leads to a separation of the Gα protein from the βγ-dimer thereby allowing the subunits to interact with their respective effector proteins [5]. Gα proteins are subdivided into four families according to their protein sequence: Gαs, Gαi, Gα₁₂/₁₃ and Gαq [6,7]. The Gαq family comprises four subclasses (Gαq, Gα₁₁, Gα₁₄, and Gα₁₅/₁₆), all of which activate phospholipase C-β (PLC-β) [8] resulting in the intracellular formation of diacylglycerol and inositol trisphosphate (IP₃) and subsequent Ca²⁺ mobilization. Further Gαq-activated effector proteins have been described, including p63RhoGEF [9], and protein kinase C zeta [10].
Despite the important role of G proteins in signal transduction, only few potent and selective pharmacological modulators are known. These include the proteins pertussis toxin (irreversible Gαi inhibitor) and cholera toxin (irreversible Gαs activator) [11,12], and the macrocyclic depsipeptides YM-254890 (YM, PubChem SID: 315495489) and FR900359 (FR, PubChem CID: 14101198), natural products that act as reversible Gαq protein inhibitors (Fig. 1A) [13–15]. The Gαq inhibitor FR has been proposed for the treatment of asthma [16,17], and of cancers that show upregulation of Gq protein expression or mutations (e.g. uveal melanoma, a malignant disease driven by constitutively active Gαq proteins [18–21]). Further disease conditions that might benefit from Gαq blockade include metabolic disorders [22], cardiac hypertrophy [23], inflammation [24], and pulmonary hypertension [25,26].
Figure 1: Chemical structures and binding poses of the macrocyclic Gαq inhibitors. (A) Chemical structures of the Gαq inhibitors YM and FR and their tritiated derivatives [³H]PSB-16254-YM and [³H]PSB-15900-FR obtained by catalytic hydrogenation of the exocyclic double bond (green) with tritium gas. The residues that differ between YM and FR and their respective radioligands are shown in red (R¹, R²), also designated “anchor 1” and “anchor 2”.

(B) Left: Model of the Gαq/i1Nβ1γ2 heterotrimer bound to GDP and FR (based on the X-ray structure of a chimeric Gαq protein, whose N-terminal helix was substituted from the Gαi1 protein (Gαq/i1N and the β1γ2 dimer bound to YM; PDB ID: 3AH8) [27,28]. The docked FR is shown as cyan sticks, the Gαq RasD is depicted in yellow, the αH domain in green, and linker 1 and switch 1 are highlighted in red. Gβ1 is depicted in light blue, Gγ2 in pink, and GDP in magenta. Right: Close-up view of the YM/FR binding site in the Gαq subunit bound to FR. The inhibitor binding site is located between the α1 helix and helix A, and the linker 1 and switch 1 (in red) regions of the Gαq protein. GDP bound to its binding site is also shown. Anchors 1 and 2 of FR are highlighted in orange. Nitrogen atoms are shown in blue, oxygen in red, and phosphorus atoms in yellow.

FR was first reported in 1988, isolated from the leaves of the plant Ardisia crenata where it is synthesized by the endosymbiotic bacteria Cand. burkholderia crenata [29,30]. YM was discovered in 2003 as a bioactive natural product synthesized by the bacterial strain Chromobacterium sp. QS3666 [31,32]. Both compounds showed inhibition of platelet aggregation and were later found to act as selective Gαq protein inhibitors [29,31]. FR and YM differ only in two substituents (Fig. 1A): FR contains a propionyl for R¹ instead of an acetyl present in YM, and an isopropyl instead of a methyl group for R². This results in an increased lipophilicity of FR as compared to YM [17]. A co-crystal structure revealed a hydrophobic pocket of the Gαq switch 1/hinge region near the Gα/β interface as the binding site of YM (Fig. 1B) [27]. Both compounds, FR and YM, act as guanine nucleotide dissociation inhibitors,
preventing the separation of the \( \Gamma \alpha_q \) helical domain (\( \alpha_H \)) from its Ras-like domain (\( \text{RasD} \)) thereby trapping the \( \Gamma \alpha_q \) subunit in its inactive GDP-bound state [13,27]. The YM/FR binding site is predominantly formed by non-polar residues, which suggests an important contribution of hydrophobic interactions to the high-affinity binding of the inhibitors (see Fig. 1B). Molecular docking studies, radioligand binding experiments, and mutational analyses suggested a virtually identical mechanism of action and very similar potency of YM and FR [13,28,33,34] leading to the suggestion that both inhibitors may be interchangeable [35]. However, we recently discovered a remarkable difference in the dissociation kinetics of an FR-as compared to a YM-derived tritium-labeled radiotracer (Fig. 1A) that translates into significantly different residence times (1/k_\text{off} or \( \tau \)) [28]. While \([^3]\text{H}\)PSB-16254-YM, obtained by catalytic tritiation of the exocyclic double bond of YM, displayed a residence time of only about 5 min, determined at 37°C, the corresponding FR-derived radiotracer \([^3]\text{H}\)PSB-15900-FR showed a more than 20-fold longer residence time of 133 min [28]. This large difference is surprising, given the very similar structure of both inhibitors (Fig. 1A). Importantly, the increased residence time translates into a significantly longer duration of pharmacological effects of FR as compared to YM, as recently demonstrated in a mouse model of asthma [17].

Classical drug development has typically focused on improving the binding affinity of a drug, i.e. the strength of the protein-ligand interaction. More recently, dissociation rates, often expressed as residence times, have emerged as another, perhaps even more important drug property [36–38]. There is increasing evidence that the unbinding kinetics of drugs may be better correlated with drug efficacy than binding affinity since the pharmacological effect of a slowly dissociating drug may still be observed after the drug has disappeared from blood circulation [39]. However, the structural determinants of residence time are largely unknown, and only a limited number of studies has been published so far [40,41]. The development of
computational methods to predict drug binding kinetics is currently hampered by the general lack of experimental data [42].

The binding mechanism of a ligand to its target is another determinant of drug action [37]. Classical pharmacological equations commonly employ a one step-binding model, where a target acts as a lock and the ligand as a key (Fig. 2, top). However, for most protein-ligand interactions, a more complex two-step binding model is assumed, requiring a conformational change of the protein, either prior to ligand binding (conformational selection; Fig. 2, bottom left) or after initial ligand binding (induced fit; Fig. 2, bottom right), to yield a high-affinity protein-ligand complex [43–45]. Some protein-ligand interactions are better described by a more complex combination of both features, e.g. selection of an initial binding conformation followed by a conformational change of the protein-ligand complex [46].
Figure 2: Models of ligand binding to a heterotrimeric G protein. (Top) Schematic depiction of a lock-and-key binding mechanism: The ligand FR or YM binds to the G protein with a concentration-dependent association rate $k_{on}[L]$ and unbinds with a concentration-independent rate $k_{off}$. No crucial conformational changes of the protein are required. (Bottom) Two-step binding models for ligand binding to heterotrimeric G proteins: In the induced-fit binding model (top right), the ligand FR or YM binds to the protein conformation $Gα'$ to form a metastable low-affinity complex $Gα'L$ driven by the concentration-dependent association rate $k_{on}[L]$, similar to the one-step binding model. The complex dissociates with the concentration-independent rate $k_{off}$. However, $Gα'L$ can be transformed to the high-affinity complex $GαL$ by a conformational change of the protein, occurring with a rate constant $k_r$. The conformational change can be reversed with the rate constant $k_{r-}$. Conversely, in conformational selection (bottom left), the protein $Gα'$ undergoes a conformational change towards the ligand-binding conformation $Gα$ prior to ligand binding. $Gα$ is formed from $Gα'$ at the rate constant $k_r$, $Gα'$ is formed from $Gα$ at the rate constant $k_{r-}$. The ligand L binds in a concentration-dependent manner only to $Gα$, but not to $Gα'$, at the rate of $k_{on}[L]$. The complex $GαL$ dissociates with the concentration-independent rate $k_{off}$.

The present study was aimed at elucidating the binding mechanism and the molecular basis for the strongly divergent dissociation kinetics of the macrocyclic $Gα_q$ inhibitors FR and YM. To this end, we introduced mutations, based on molecular modeling, and expressed the resulting $Gα_q$ protein mutants in human embryonic kidney (HEK) 293 cells in which the native $Gα_q$ proteins had been knocked out by CRISPR-Cas9. As previously observed for native platelet membranes, [$^3H$]PSB-15900-FR dissociated very slowly from the recombinant $Gα_q$ subunit expressed in HEK cells, while [$^3H$]PSB-16254-YM displayed fast dissociation. While most of the investigated $Gα_q$ protein mutants retained high potency and affinity, dissociation kinetics of the inhibitors, in particular those of the FR-radioligand, were strongly accelerated. Especially
mutations around the binding site of the isopropyl residue of FR (R², anchor 2) led to rapid
dissociation of the inhibitor-protein complex and thus to a strongly reduced residence time. Our
results emphasize the importance of investigating the binding kinetics of protein-drug
interactions.
2. Materials and Methods

2.1. Bioactive Compounds

FR was isolated and purified from *A. crenata* leaves in the laboratory of G. M. König as previously described [13]. YM was purchased from Wako Chemicals (Neuss, Germany). The radioligands \[^3\text{H}]\text{PSB-15900-FR}\) (FR-derived, specific activity: 1036 GBq*mmol\(^{-1}\)/28 Ci*mmol\(^{-1}\)) and \[^3\text{H}]\text{PSB-16254-YM}\) (YM-derived, specific activity: 1147 GBq*mmol\(^{-1}\)/31 Ci*mmol\(^{-1}\)) were synthesized by catalytic hydrogenation of FR and YM, respectively, with tritium gas (Pharmaron, Cardiff, UK) as previously described [28].

2.2. Docking studies

The docked pose of FR in complex with the trimeric G\(\alpha_q\beta_1\gamma_2\) protein was obtained as previously described [28]. The protein structure (PDB-ID: 3AH8) [27] was prepared in MOE 2016.08 (Chemical Computing Group, Montreal, Canada). AutoDock 4.2 was used to dock FR into the inhibitor binding site [47]. Atomic partial charges were calculated by AutoDock Tools [47]. Based on the position of the co-crystallized ligand YM in the X-ray structure, three-dimensional energy scoring grids of 60 x 60 x 60 points were computed with a spacing of 0.375 Å. During docking, which was performed by the *var*CPSO-Is algorithm from PSO@Autodock implemented in AutoDock 4.2 [48], FR was fully flexible, while the G protein remained rigid. We performed 50 independent docking calculations, terminated after 500,000 evaluation steps. The cognitive and social coefficients c1 and c2 of the *var*CPSO-Is algorithm were set at 6.05 with a swarm size of 60 individual particles; all other parameters remained at default. The depicted pose was selected based on lowest binding energy and visual inspection of the inhibitor-protein interactions. All docking images were created with PyMOL (Schrodinger, New York, USA).

2.3. Site-directed mutagenesis
The coding sequence of murine Gnaq (see UniProt ID P21279 for protein sequence) was obtained in pcDNA3.1(+), in which we performed site-directed mutagenesis. The sequence was modified to contain a partial internal HA-tag (DVPDYA; required mutations: E125D, N126V, Y128D, V129Y, and D130A) between residues 125-130. Single amino acid exchanges were induced by whole-plasmid polymerase chain reaction (PCR; 150 ng template DNA, 0.25 mM dNTP mixture, 0.25 µl DMSO, 5 µl GC-buffer, 125 nM each forward- and reverse primer, 0.5 µl Phusion polymerase (New England Biolabs, Ipswitch, MA, USA); thermocycling protocol: 30 s 98°C, 20 cycles of 10 s 98°C, 30 s 58°C, 5 min 72°C, 10 min final elongation at 72°C) using a set of specific primers, listed in Supp. Table 1. Subsequently, competent DH5α E. coli were transformed with DpnI-digested (New England Biolabs; 90 min, 37°C) PCR product and added onto Ampicillin-containing agar plates. The next day, cDNA was isolated from individual clones and the DNA sequence was confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany). cDNA was amplified and cloned into the retroviral expression vector pQCXIN.

2.4. Cell culture

HEK293 cells (human, female), whose GNAQ/GNA11 genes were previously removed via CRISPR/Cas9 (HEK293 Gaq/11-KO; obtained from A. Inoue) [13,49], were cultured at 37°C, 5% CO2 in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and a penicillin/streptomycin mixture (PenStrep; final concentrations: penicillin = 100 U*ml⁻¹, streptomycin = 0.1 mg*ml⁻¹). Medium for HEK293 Gaq/11-KO cells recombinantly expressing Gaq protein was additionally supplemented with G418 (0.2 mg*ml⁻¹). When cells were about 70-80% confluent, the medium was discarded and the cell monolayer was rinsed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄). Subsequently, cells were incubated in a trypsin-ethylenediaminetetraacetic acid (EDTA) solution at room temperature, detached from the cell
culture flask, and diluted into new cell culture flasks. Cell lines were routinely checked for mycoplasma contamination by PCR; cell lines used for membrane preparations and calcium mobilization assays were passaged less than 10 times after retroviral transfection.

2.5. Retroviral transfection

HEK293 Gaq11-KO cells were stably transfected with the coding sequence for either wild-type (wt) or mutant murine Gnaq as previously described for the P2Y2 receptor [50]: On the first day, 1.5 x 10^6 GP’envAM12 packaging cells were seeded and transiently transfected with the Gnaq cDNA (in the pQCXIN vector, 6.25 µg) and vesicular stomatitis virus G (VSV-G, in pcDNA3.1, 3.75 µg) protein cDNA on day 2, utilizing lipofectamine 2000 (25 µl). After 16 h, production of viral vectors was induced by the addition of 3 ml DMEM containing 10% FBS, PenStrep and 5 mM sodium butyrate, followed by a 48 h incubation at 32°C. Viral vectors were harvested, filtered (0.2 µm pore diameter), and added to HEK293 Gaq11-KO cells. Polybrene solution (6 µl, 4 mg*ml^-1 in water, sterile filtered) was added. After 2.5 h, the virus solution was discarded and the cells were supplemented with fresh DMEM + 10% FCS + PenStrep. Selection for geneticin resistance was induced 72 h later by the addition of 0.2 mg/ml G418 to the media.

2.6. Cell membrane preparations

Recombinant HEK293 cells were cultured as described above and kept in an exponential growth phase. To obtain membrane preparations, HEK293 cells were seeded into cell culture dishes. When the cell monolayer became confluent, the medium was discarded, and the dishes were frozen at -20°C overnight. After defrosting, cells were detached with a rubber scraper while adding 5 mM Tris + 2 mM EDTA, pH 7.4. The cell suspension was homogenized with an UltraTurrax® (IKA Labortechnik, Staufen, Germany) for 1 min at level 4. The homogenate was centrifuged for 10 min at 1,000 g, 4°C; the pellet (P1) was discarded and the supernatant (S1) was then centrifuged for 1 h at 48,400 g, 4°C. The supernatant (S2) was discarded, the
pellet (P2) was resuspended in 5 mM Tris + 2 mM EDTA, pH 7.4, and centrifuged for another
hour at 48,400 g, 4° C. Again, the supernatant was discarded, the pellet resuspended in 50 mM
Tris-HCl, pH 7.4, and aliquots were stored at -80° C until use.

2.7. Protein determination

Protein concentration was determined by the Lowry method [51]: 2% (w/v) NaHCO₃ were
dissolved in NaOH; this solution was mixed 50:1 with an aqueous 0.5% (w/v) Cu₂SO₄ + 1%
(w/v) sodium tartrate solution. Of this mixture, 1 ml was added to 200 µl of sample solution
diluted 1:20 in Tris buffer in 50 mM Tris buffer, pH 7.4. After incubation for 20 min at room
temperature, 100 µl Folin phenol reagent solution (18 ml Folin reagent in 90 ml distilled water)
were added to the mixture, which was then vortexed thoroughly. After another 30 min of
incubation at room temperature, absorption was measured at 500 nm. A bovine serum albumin
(BSA) calibration curve from 0.1 to 0.5 mg BSA/ml served for calibration.

2.8. SDS-PAGE and Western blotting

HEK293 cell membrane preparation (20 µg of protein) was mixed with 4x sodium dodecyl
sulfate (SDS) loading buffer, and the mixture was diluted with H₂O to a final volume of 20 µl.
Samples were heated at 95° C for 5 min and loaded onto a 10% acrylamide Tris-glycine gel.
Proteins were separated by applying a constant voltage of 200 V for approximately 1 h and then
transferred to a nitrocellulose membrane by tank blotting (75 V constant voltage, 90 min, in
200 mM glycine + 20 mM Tris + 20% MeOH on ice). Successful protein transfer was confirmed
by an in-between Ponceau S staining of the nitrocellulose membrane; afterwards non-specific
binding sites were blocked with PBS + 0.1% Tween 20 (PBS-T) + 5% milk powder (blocking
buffer). Prestained PageRuler™ protein ladder (6 µl; ThermoFisher, Waltham, MA, USA)
served as a protein standard. Recombinant expression of the Gαq protein was detected by its
internal HA-tag. A primary murine antibody against the HA-epitope (BioLegend, Cat. Nr.
901502) was diluted in blocking buffer and the membrane was incubated at 4° C overnight. The
next day, the primary antibody solution was discarded, and the membrane was rinsed and washed with blocking buffer 4 times every 5 minutes. A secondary horseradish peroxidase (HRP)-coupled goat anti-mouse antibody (Jackson ImmunoResearch, 115-035-003, Lot No.: 146779; 1:4000 in blocking buffer) was incubated with the membrane for 90 min at room temperature. After washing the membrane five times for 5 min each step with PBS-T, it was covered with a luminol-peroxide detection reagent (ThermoFisher, Waltham, MA, USA). Chemiluminescence was detected by a multi-purpose imager (BioRad ChemiDoc, BioRad, Wien, Austria).

2.9. Determination of specific binding and competition binding assays

Radioligand binding assays were performed in 50 mM Tris-HCl, pH 7.4, in a final assay volume of 200 µl as previously described [28]. Wt or mutant HEK293 membrane preparations (50 µg of protein if not indicated otherwise) were co-incubated with ~5 nM of the respective radioligand for 90 minutes at 37°C with gentle shaking. Total binding of the radioligand was determined in the presence of 5 µl dimethyl sulfoxide (DMSO) and non-specific binding was determined in the presence of FR or YM, respectively, dissolved in 5 µl DMSO (final concentration: 5 µM). In competition binding assays, different concentrations of unlabeled FR or YM, dissolved in DMSO, were added to the mixture.

Incubation was terminated by rapid filtration through GF/C glass-fiber filters using a Brandel 24-well harvester. Assay tubes were rinsed thrice with cold Tris-HCl + 0.1% BSA + 0.1% Tween20. Filters were dried, punched out, and transferred to scintillation vials. Luma Safe® scintillation cocktail (2.5 ml) were added and the filters were incubated for at least 9 h prior to measurement in a liquid scintillation counter (53-55% counting efficiency). Non-specific binding was subtracted from total binding to determine specific binding. Results are depicted in pmol bound radioligand per mg protein.

2.10. Calcium mobilization assays
Inhibition of G\(_q\)‐mediated calcium mobilization by FR and YM in recombinant HEK293 cells was determined in analogy to described procedure [50,52]. One day prior to testing, HEK293 cells were detached from their cell culture flasks and seeded into black clear-bottom 96-well plates (Corning 3340) at a density of 45,000 cells per well. The following day, the medium was discarded and cells were incubated for 1 h with a mixture of fluo-4-acetoxymethyl ester (final concentration: 3 \(\mu\)M) and Pluronic F-127 (final concentration: 0.075% (w/v)), both dissolved in DMSO (final DMSO concentration at this step: 0.6%) in Hank’s balanced salt solution (HBSS). All incubation steps were performed at 25° C with gentle shaking of the plates. After removing excess dye, 178 \(\mu\)l HBSS + 2 \(\mu\)l FR/YM solution in DMSO were added to the cells, which were then incubated for 30 min. For activation, 20 \(\mu\)l of a solution of a GPCR agonist (ATP corresponding to an EC\(_{80}\) concentration if not mentioned otherwise), dissolved in HBSS, was pipetted into each well and fluorescence at \(\lambda = 525\) nm was measured by a NovoStar microplate reader (BMG Labtech, Offenburg, Germany). As a positive control, cells were incubated with pure DMSO solution in the last step; in negative controls, cells were co-incubated with pure DMSO and stimulated with HBSS only.

### 2.11. Binding kinetics

Assays were performed in 50 mM Tris-HCl, pH 7.4, in a final assay volume of 200 \(\mu\)l. For association, HEK293 membrane preparation (50 \(\mu\)g of protein) was mixed with 95 \(\mu\)l of buffer and 5 \(\mu\)l of DMSO. Non-specific binding was determined in the presence of 5 \(\mu\)l YM or FR (final concentration: 5 \(\mu\)M) in DMSO. The mixture was incubated at 37°C in a water bath upon gentle shaking. At given time points, 50 \(\mu\)l of radioligand solution was added to the mixture. Further assay handling was done as described above for the determination of specific binding. Specific binding of the radiotracers was determined, results were normalized to 0 cpm = 0% and specific binding at time 0 = 100%. Association half-life was calculated by the “one phase - association”-equation, implemented in GraphPad Prism 7.0 (GraphPad, San Diego, CA):
Y=Y_{\text{max}}[1-\exp(-k_{\text{obs}}*X)], with t_{1/2} = \ln(2)/k_{\text{obs}}. Observed association rates were not corrected for dissociation.

For dissociation experiments, 95 µl of buffer, 50 µl of HEK cell membrane suspension (50 µg of protein) and 50 µl of radioligand (final concentration: 5 nM) were pre-incubated for 60 min at 37°C to reach equilibrium. Dissociation was initiated at given time points by adding 5 µl of YM or FR in DMSO (final concentration: 5 µM). The subsequent assay procedure was the same as described above. Specific binding was calculated and normalized as described above for association. Normalized data were fit with the “one phase exponential decay”-equation (Y=(Y_{0}-NS)*\exp(-k_{\text{off}}*X)) in GraphPad Prism 7.0 and the dissociation t_{1/2} was calculated (t_{1/2} = \ln(2)/k_{\text{off}}).

### 2.12. Molecular dynamics simulations

VMD1.9.4 [53] was used to preprocess the crystal structure of the G\alpha_{q11N}\beta1\gamma2 protein in complex with YM-254890 (PDB ID:3AH8). Any co-crystallization atoms different than water molecules closer than 5 Å to the protein were removed. Missing G\alpha N- and C-terminal residues 2 to 6 and 355 to 359 were modelled ab initio using MODELLER (version 10.1) [54]. MODELLER was also employed to model via homology modelling both the G\beta N-terminal residues 2 to 10, and the G\gamma N- and C-terminal residues 2 to 10 and 61 to 67. The FR-bound structure was generated by superposing FR onto YM, and subsequently removing the latter. The inhibitor-free structure was generated by simply removing YM from the binding pocket. Each inhibitor-bound and the inhibitor-free structure was then placed into a water box made of explicit water molecules, and the global electrostatic charge and ionic strength was adjusted using the CHARMM-GUI builder [55]. All titratable residues of the protein were left in their dominant protonation state at pH 7.0. The solvated system was first geometry-optimized and subsequently relaxed by applying harmonic positional restraints to all C\alpha atoms of the protein that were gradually released throughout the equilibration. The first step of the equilibration
phase was run for 0.125 ns, the second step was run for 10 ns. Three independent trajectories were spawned from the last snapshot of the equilibrated system using a random seed. Production simulations for each replica were run in the NVT ensemble at 1,013 bar and 310 K for 500 ns. The production simulations of this study yielded an aggregated time of 4.5 µs (3 systems x 3 replicas x 500 ns). All simulations were run using GROMACS v2020.4 [56] in combination with the CHARMM36m force field [57]. Figures from simulations were rendered using the Tachyon renderer [58].

### 2.13. Quantification and statistical analysis

For each data point, at least three individual experiments (n ≥ 3) were performed, each in duplicate. The exact number of replicate experiments can be found in figure and table legends. Unless otherwise stated, data are expressed as mean ± SEM.

Statistical analysis was carried out by using GraphPad Prism v. 7.0. To assess a mean difference between two groups, an unpaired t-test was employed when data displayed normality and variance homogeneity. When data showed variance inhomogeneity, Welch’s t-test was used instead. Normality and variance homogeneity were assessed with Shapiro-Wilk’s test and F-test, respectively. To determine significant differences of a mean in a series of three or more experimental conditions, a one-way analysis of variance (ANOVA) was carried out. If the difference among means was significant (p<0.05), the mean of each column was compared with the mean of the reference condition and corrected for multiple comparisons using Dunnett’s post-hoc test. To compare the difference between two groups (YM and FR) under multiple different conditions (several Goαq mutants), multiple unpaired t-tests were used and corrected for multiple comparisons according to the Holm-Sidak-method. P-values of <0.05 were considered to be statistically significant (*), p<0.01 was considered to be very statistically significant (**) and p<0.001 was considered to be highly statistically significant (***)

### 2.14. Data and material availability statement
All data sets obtained in this study, retrovirally transfected HEK293 cell lines, mutated and wild-type *Gnaq* coding sequence in pcDNA3.1(+) and pQCXIN are available upon reasonable request by a qualified researcher. Radiolabeled compounds can only be made available to researchers with an appropriate handling license, and the access to those compounds may be limited due to radioligand batch size.
3. Results

3.1. Expression and characterization of the wildtype Gαq protein

As a first step, we expressed and comprehensively characterized the wt Gαq protein expressed in HEK293 cells whose GNAQ/GNA11 genes had been knocked out by CRISPR-Cas9. To confirm recombinant expression of the Gαq protein, we determined binding of [³H]PSB-15900-FR and [³H]PSB-16254-YM to wt Gαq-expressing HEK293 cell membrane preparations (Fig. 3A). In CRISPR-Cas9 knockout (KO) cells transfected with the wt Gαq protein we observed high specific binding of both radioligands, while no specific binding was detected in KO cells lacking Gαq proteins. To estimate Gαq protein expression levels and apparent dissociation constants (K<sub>D</sub> values), saturation experiments were performed (Fig. 3B for [³H]PSB-15900-FR, Fig. 3C for [³H]PSB-16254-YM). Binding was saturable, and the following values were determined: [³H]PSB-15900-FR, pK<sub>D</sub> = 7.92 ± 0.12, B<sub>max</sub> = 19.8 ± 2.9 pmol/mg protein; [³H]PSB-16254-YM, pK<sub>D</sub> = 7.80 ± 0.09, B<sub>max</sub> = 21.8 ± 4.7 pmol/mg protein. There was no significant difference between apparent pK<sub>D</sub> and B<sub>max</sub> values obtained with either radioligand, [³H]PSB-15900-FR or [³H]PSB-16254-YM, confirming previous results on human platelet membranes [28].

Next, we studied binding kinetics. Association of both radioligands was rapid showing almost identical association kinetics ([³H]PSB-15900-FR: association t<sub>1/2</sub> = 2.18 ± 0.21 min; [³H]PSB-16254-YM: association t<sub>1/2</sub> = 2.47 ± 0.57 min; see Fig. 3D). However, dramatic differences in dissociation kinetics and thus in residence times of both radioligands were observed in recombinantly expressed wt Gαq proteins, consistent with those previously determined in native human blood platelet membranes [28]. In wt Gαq proteins, [³H]PSB-15900-FR displayed a long dissociation half-life of 79.2 ± 1.54 min determined at 37°C, while [³H]PSB-16254-YM showed a substantially shorter half-life of only 3.89 ± 0.16 min (Fig. 3E). The calculation of kinetic K<sub>D</sub>...
values ($k_{off}/k_{on}$) performed under the assumption of a one-step binding model (using the
equation $k_{obs} = k_{on}[L]+k_{off}$; $k_{obs} = \ln(2)/\text{association t}_{1/2}$; [L] radioligand concentration, $k_{off} =$
$\ln(2)/\text{dissociation t}_{1/2}$) resulted in kinetic pK$_D$ values of 9.85 for $[^3H]$PSB-15900-FR and of 8.06
for $[^3H]$PSB-16254-YM.

Next, we examined the dependence of $k_{obs}$ on ligand concentration, [L], by performing
association experiments with varying concentrations of $[^3H]$PSB-15900-FR (Fig. 3F) and
[^3H]PSB-16254-YM (Fig. 3G). Assuming a one-step binding model, $k_{obs}$ would linearly
increase with increasing ligand concentration [L]. The obtained data, however, fitted best to an
exponentially decaying curve resulting in a Y-axis intercept of 0.64 min$^{-1}$ and a plateau of 0.32
min$^{-1}$ for $[^3H]$PSB-15900-FR, and a Y-axis intercept of 0.65 min$^{-1}$ and a plateau of 0.22 min$^{-1}$
for $[^3H]$PSB-16254-YM. This indicates a binding mechanism that is based on conformational
selection (described in Fig. 2).
Figure 3: Binding of FR-derived radioligand [³H]PSB-15900-FR and YM-derived radioligand [³H]PSB-16254-YM to HEK cell membrane preparations recombinantly expressing the wt Ga₉ protein. (A) Specific binding of [³H]PSB-15900-FR and [³H]PSB-16254-YM (5 nM each) to HEK293 Ga₉-WT membrane preparations (50 µg of protein) in the presence or absence of recombinantly expressed Ga₉ protein. Incubation was performed for 90 min at 37°C. (B, C) Saturation binding curves of [³H]PSB-15900-FR (B) and [³H]PSB-16254-YM (C) to HEK293 membrane preparations (25 µg of protein) recombinantly expressing wt Ga₉ protein, incubated at 37°C for 90 min. (D) Association of [³H]PSB-15900-FR or [³H]PSB-16254-YM (5 nM each) to HEK293 cell membrane preparations recombinantly expressing wt Ga₉ protein (50 µg of protein) at 37°C. (E) Dissociation of [³H]PSB-15900-FR and [³H]PSB-16254-YM (5 nM each) from wt Ga₉ protein-expressing HEK293 membrane preparations (50 µg of protein) at 37°C.
Dissociation was induced by the addition of 5 µM of FR, or YM, respectively, after a 60 min of pre-incubation with the radioligand. (F, G) Plots of $k_{\text{obs}}$ (min$^{-1}$) versus [L] (nM) for [$^3$H]PSB-15900-FR (F) and [$^3$H]PSB-16254-YM binding (G). Association experiments were performed at 37°C with wt Gαq HEK293 cell membrane preparations (10 µg of protein). Y-axis intercept (labeled as $k_r + k_{-r}$) and plateau levels (labeled as $k_r$) are indicated by dashed lines. Data points represent mean ± SEM of at least three independent experiments performed in duplicates (A, D-F). In panels B and C, a representative trace out of three individual experiments performed in duplicate is depicted; in panels G and H, each data point represents an individual experiment.

3.2. Site-directed mutagenesis study to reveal molecular determinants for the long residence time of FR

As a next step, we set out to study the molecular basis for the large difference in residence time between both radiotracers. The substituents R$^1$ and R$^2$ (Fig. 1) represent the only differences between FR and YM (and their radiolabelled derivatives) and must therefore be ultimately responsible for the large difference in dissociation kinetics. We hypothesized that the larger and more lipophilic substituents, ethyl (R$^1$) and isopropyl (R$^2$) in FR and its radioligand [$^3$H]PSB-15900-FR, may form stronger hydrophobic interactions with the Gαq protein than the methyl groups in the corresponding positions of YM and its radioligand [$^3$H]PSB-16254-YM. These residues in FR might act as anchors locking the inhibitor in its binding site [28]. To analyze the molecular basis for the differences in residence time, we modified the inhibitor binding site [27,28] in the Gαq protein by site-directed mutagenesis. We focused on residues presumed to interact with “anchor 1” (R$^1$), and “anchor 2” (R$^2$), respectively (see Fig. 1B and Fig. 4).
Figure 4: Visualization of the residue exchanges near anchor 1 and anchor 2. (A) Surface representation and (B) stick model of the wt Gαq inhibitor binding site (yellow) in complex with the docked pose of FR (cyan, anchors 1 (ethyl residue, top) and 2 (isopropyl residue, bottom) are highlighted in orange). (C, D) Visualization of all mutations evaluated in binding assays at the Gαq binding site, highlighted in magenta. Residues subjected to mutagenesis during the study are labeled in black, mutated residues in (D) are labeled in magenta, residues not investigated in the course of the study are labeled in gray. The protein structure is based on the co-crystal structure of the heterotrimeric Gαqβγ2 protein in complex with GDP and YM; PDB-ID: 3AH8 [27].
Anchor 1 of FR is proposed to bind to a rather wide interface [28] between Gβ and the β2/β3 strands of Gaq (residues I190G.S02.02, E191G.S02.03, P193G.S02.05 and R202G.S03.04, Fig. 1B and Fig. 4; residues are labeled according to the CGN numbering system for heterotrimeric G proteins [4]). The predicted binding mode potentially allows for significant conformational flexibility of anchor 1. We generated a series of point mutants in this region of the Gaq protein. Mutants I190F and I190W are expected to disrupt a small hydrophobic cluster that likely forms crucial hydrophobic interactions with FR [15]. Mutant E191A disrupts a salt bridge with R202G.S03.04 that stabilizes the β2/β3 sheet (see Fig. 4). The R202H mutant preserves the charge of the side chain but alters its size introducing steric hindrance. Finally, mutant P193C (inspired by the YM/FR-resistant Ga15/16 [33], which harbors the same amino acid exchange) was designed to alter the structure of the β2 strand. We expected that these alterations of the YM/FR binding pocket near anchor 1 would provide information on the molecular determinants of their binding interactions.

In anchor 2, the lipophilic isopropyl group of FR is predicted to contact residues in helix A of the αH Ga domain (G74H.HA.06, F75H.HA.07, L78H.HA.10, see Fig. 4). In this region, we generated a series of mutants meant to directly disrupt these interactions. The small G74H.HA.06 was replaced by a bulky valine residue (G74V), whose side chain may clash with the isopropyl moiety of FR. F75H.HA.07 was exchanged to alanine (F75A) to disrupt its predicted interaction with FR, and to lysine (F75K), respectively, to further disrupt the hydrophobic cluster between linker 1 and switch 1 [15], which was expected to perturb inhibitor binding. L78H.HA.10 was mutated to alanine (L78A) to eliminate another potential hydrophobic interaction partner for FR (see Fig. 4). In addition, we prepared the double mutant G74V/L78A, since both mutations were expected to affect the interaction of the isopropyl group in FR with the Gq protein. Finally, the exchange of V184G.hfs2.03 (in switch 1) to methionine (V184M) was again inspired by the YM/FR-resistant Ga15/16 mutant [33]. Although methionine is also lipophilic, its higher
3.3. Characterization of mutant Gaq proteins confirms binding of inhibitors

Mutations to the Gnaq gene were introduced by site-directed mutagenesis into the pcDNA3.1(+) vector. Cloning and expression were performed as described for the wt Gaq protein (see sections 2.3, 2.5, and 3.1) to obtain membrane preparations of recombinant HEK293 Gαq/11-KO cells expressing the designed mutants. All mutated proteins were found to be expressed in similar quantity with the exception of the E191A and I190W mutants, which showed lower expression levels (Fig. 5A; see Supp. Fig. 1 for full blots). We were unable to stably express the F75A mutant in HEK293 cells despite multiple attempts of retroviral transfection.

Next, we measured specific binding of both radioligands to HEK cell membrane preparations recombinantly expressing the mutant Gaq proteins (Fig. 5A). Membrane preparations expressing the Gaq mutants F75K, I190F, and I190W could not be labeled by the radioligands indicating that they had lost high affinity binding. In contrast, mutants E191A, P193C, R202H, G74V, L78A, V184M, and G74V/L78A all bound [3H]PSB-15900-FR and [3H]PSB-16254-YM with high affinity, although specific binding was notably lower than at the wt Gq protein, ranging between 10-54% ([3H]PSB-15900-FR) and 4-33% ([3H]PSB-16254-YM) (Fig. 5A). Both radioligands displayed only low specific binding at the G74V/L78A Gq protein mutant. Overall, [3H]PSB-16254-YM showed somewhat lower specific binding at the mutant Gaq proteins than [3H]PSB-15900-FR (Fig. 5A) determined under the same conditions.
Figure 5: Characterization of mutant Gαq proteins recombinantly expressed in HEK293 Gαq/11-KO cells. (A) Top: Specific binding (% of wt) of [³H]PSB-15900-FR (5 nM) to HEK293 cell membrane preparations recombinantly expressing Gαq subunits (50 µg of protein) after 90 min of incubation at 37°C. Bottom: Western blot images of HEK293 Gαq/11-KO cell membranes recombinantly expressing Gαq proteins (20 µg protein per lane). A primary antibody against the HA-tag was used to detect recombinant Gαq proteins; HEK293 Gαq/11-KO cell membrane preparations served as a negative control. The expression level is indicated as follows: (−) no expression, (+) low expression, (++) moderate expression, (+++) high expression. (B) Pseudo-pKᵦ values [28] calculated by pseudo-homologous competition binding assays of YM versus [³H]PSB-16254-YM. (C) Potency (pIC₅₀) of FR (red) and YM (blue) in HEK293 cells expressing mutated Gαq protein subunits compared to the wt Gαq protein. Calcium mobilization was induced by an EC₈₀ concentration of ATP. Multiple t-tests were employed to compare the
means between YM and FR of individual mutants; significant differences are indicated (#). The
potency of YM and FR at all Gαq mutants was tested for significant differences from wt Gαq
protein with a one-way ANOVA, followed by Dunnett’s post-hoc test; significant differences
(p < 0.05) are indicated with asterisks. All data represent means ± SEM of at least three separate
experiments performed in duplicates.

Subsequently, the Gαq mutants were characterized in competition binding and functional assays
to determine their affinity for FR and YM, their functionality, and the compounds’ inhibitory
potency. The pseudo-pK_D values of the mutants were determined using a competition binding
assay of YM versus [³H]PSB-16254-YM. We selected this radioligand due to its faster
dissociation kinetics in wt Gαq proteins; therefore, it was easier to handle and did not require
ultra-long incubation times to reach equilibrium. In the wt Gαq protein, the pseudo-pK_D value
determined for YM was 8.19; all mutant Gαq subunits that could be investigated in binding
studies showed similar values not significantly different from wt (i.e., all pseudo-pK_D values
were around 8, see Supp. Table 4 and Fig. 5B).

Next, the inhibitory potencies of YM and FR were determined by GPCR-induced calcium
mobilization assays in HEK293 cells expressing either the wt or a mutant Gαq protein. In
preliminary experiments, we tested the potency and efficacy of several agonists of Gq protein-
coupled GPCRs that are natively expressed in HEK cells. The nucleotides UDP, UTP, ADP,
and ATP, and the acetylcholine analog carbachol all led to calcium mobilization in cells
transfected with the wt Gαq protein (Supp. Fig. 2A), while Gαq11-KO cells did not respond to
any of these agonists (Supp. Fig. 2B). For subsequent experiments, we selected ATP,
presumably acting via the P2Y_11 receptor endogenously expressed in HEK293 cells [59],
because it provided the largest signal window. All mutants were found to be fully responsive to
ATP (comparable to wt Gαq HEK293 cells) and displaying quantitatively similar calcium
mobilization (see Supp. Fig. 2C). Both YM and FR (1 µM) were able to completely block ATP-
induced calcium mobilization in the cell line expressing the wt Gαq protein as well as in most of the cell lines expressing mutant Gαq proteins. YM and FR had very similar potencies at the wt Gαq protein (pIC<sub>50</sub>(FR) = 8.20, pIC<sub>50</sub>(YM) = 8.09) and most of the mutant Gαq proteins (Fig. 4B). Exceptions were the Gαq mutants F75K, I190F, and I190W, which also had not shown high affinity binding in radioassays. For these mutants higher concentrations of YM and FR were required to inhibit ATP-induced calcium mobilization. YM and FR did not display significant differences in potency at each individual Gαq mutant, with the exception of the I190W mutant, where YM was less potent than FR (pIC<sub>50</sub> = 7.73 for FR and 6.16 for YM; all pIC<sub>50</sub> values are summarized in Supp. Table 3).

3.4. Kinetic studies reveal drastically reduced residence times of FR at Gq protein mutants

As a next step, we measured the binding kinetics of both radioligands and all of the mutants displaying sufficiently high radioligand binding. Association and dissociation rates of the radioligands (5 nM) at the recombinant HEK293 cell membrane preparations were determined at 37°C, and observed association half-lives (t<sub>1/2</sub> = ln(2)/k<sub>obs</sub>) were calculated. In most cases, both radiotracers displayed fast association to the mutant Gαq subunits (Fig. 6A, B), similar as to wt Gαq protein (t<sub>1/2</sub> [³H]PSB-15900-FR = 2.18 min; t<sub>1/2</sub> [³H]PSB-16254-YM = 2.47 min; see also Fig. 3D). One exception was the E191A mutant, that exhibited a significantly slower association half-life of 7.71 min for [³H]PSB-16254-YM, but not for [³H]PSB-15900-FR (3.22 min)). Moreover, [³H]PSB-15900-FR bound to the P193C and to the G74V/L78A double mutant somewhat faster than to the wt Gαq protein (t<sub>1/2</sub> = 1.21 min for P193C and 1.11 min for G74V/L78A vs. 2.18 min for wt Gαq protein, see Supp. Table 5). All other determined association half-lives of both radioligands at the mutant Gαq proteins were not significantly different from those observed for the wt Gαq protein.
In contrast to the fast association, dissociation, in particular that of the slowly dissociating FR-derived radioligand, was strongly affected by the mutations in the binding sites (Fig. 6C,D, see Supp. Fig. 3 and 4 for dissociation curves and Supp. Table 6 for calculated values). For reference, in wt Gαq-expressing cell membranes [³H]PSB-15900-FR dissociated with a half-life of ~80 min at 37°C, while [³H]PSB-16254-YM dissociated about 20-fold faster, with a half-life of only ~4 min. All mutations in our study led to significantly accelerated dissociation of [³H]PSB-15900-FR. The R202H mutant showed the least effect with a decrease of the dissociation half-life from 80 to 60 min. All other mutations led to greatly reduced dissociation half-lives of below 10 min; the “anchor 2-mutants” G74V and G74V/L78A even caused [³H]PSB-15900-FR to dissociate with a half-life of less than 1 min, accelerating the dissociation by 88- and 198-fold, respectively. The effects of the mutants on the dissociation rate of [³H]PSB-16254-YM were qualitatively similar, but much less dramatic, since its dissociation determined at the wt Gαq protein was already fast (t½ ~ 4 min). [³H]PSB-16254-YM displayed a dissociation half-life of only ~1 min at the E191A, P193C, G74V, and L78A mutants, which is approximately 4-5 times faster compared to that at the wt Gαq protein. The mutants V184M and G74V/L78A, located near “anchor 2”, showed the fastest dissociation rate for [³H]PSB-16254-YM resulting in half-lives of 0.37 and 0.30 min, respectively, which is more than 10 times faster than their dissociation from wt Gαq protein.

With the assumption of a one-step binding model, pKₐ values for the rapidly dissociating mutants (kₐ > kₐ) could not be calculated with the exception of the following radioligand – Gαq mutant combinations: [³H]PSB-15900-FR binding to Gαq L78A (pKₐ = 8.92), V184M (7.84), P193C (8.82), and R202H (9.88) and [³H]PSB-16254-YM binding to Gαq R202H (7.77).
Figure 6: Binding kinetics of Gαq radiotracers at HEK293 Gαq/11-KO cells recombinantly expressing mutant Gαq proteins. (A) Association half-lives (ln(2)/k<sub>obs</sub>) of [³H]PSB-15900-FR, (B) association half-lives of [³H]PSB-16254-YM, (C) dissociation half-lives (ln(2)/k<sub>off</sub>) of [³H]PSB-15900-FR, (D) dissociation half-lives of [³H]PSB-16254-YM. All experiments were conducted with 5 nM of radioligand and 50 µg of protein at 37°C. Data are presented as means ± SEM of three or four independent experiments each performed in duplicate (A-D).

3.5. Molecular dynamics simulations propose mechanism for conformational selection
In the past decade, advanced computational approaches have emerged to predict binding kinetics of ligand-target interactions [60,61]. Some of these approaches are based on enhanced molecular dynamics methods that simulate the process of ligand binding/unbinding at the atomic level. However, due to the potential conformational heterogeneity of the macrocyclic depsipeptides and of the Gαq binding site (which includes the flexible linker 1 and switch 1 regions), a very thorough sampling of the conformational landscape during the binding/unbinding process is required to predict binding kinetics of these inhibitors with accuracy. In this study, in order to gain insights into the inhibitor-Gq interactions at the atomic level, we settled to perform unbiased molecular dynamics simulations of the Gq-FR and Gq-YM complexes and of the inhibitor-free Gq protein. We observe stable binding poses for both inhibitors, and how the base of helix A in the αH domain (D69H.HA.01 - L78H.HA.10) of Gαq shapes the inhibitor binding site, stabilizing several key hydrophobic interactions with anchor 1 of FR. Our simulations of the inhibitor-free Gq allowed us to explore the conformational landscape around this region of the binding site (Fig. 7A). We observed that, in the absence of an inhibitor, helix A in the αH domain of Gαq is dynamic and can swing between two extreme conformations. A population of this ensemble (red ribbons in Fig. 7A, left panel) results in a wider binding site suitable for inhibitor high-affinity binding (Fig. 7A, right panel). While the molecular mechanism of Gq inhibition by YM/FR is certainly more complex, we speculate that relocation of A in the αH domain of Gαq observed in our simulations is part of the conformational selection mechanism of these inhibitors (Fig. 7B).
**Figure 7:** Effect of FR/YM binding on the conformational dynamics of helix A in the αH domain of Gα<sub>q</sub>. (A) The αH domain is shown as green cartoons, with time evolution across the simulation depicted on helix A (ribbons) in a blue-to-red gradient (blue – beginning of the simulation; red – end of the simulation) that colors 40 frames evenly distributed across the complete trajectory of a representative replica. The Gβ subunit is shown as blue cartoons and a translucent molecular surface. Except for helix A, only the first frame of the simulation is shown. In the absence of the inhibitor (left), the base of helix A can tilt towards the Gβ subunit thereby significantly obstructing the entrance route to the binding pocket (red ribbons); in this ‘closed’ conformation, residues at the base of helix A would clash with the inhibitor binding pose (crystal pose (A) or initial frame of the simulations (B), shown as a translucent surface and orange sticks for reference). Inhibitors may select the open state of helix A (blue in the left panel) to bind with high affinity (right), thus preventing the inward tilt of helix A. (B) Proposed binding mechanism of YM and FR to the Gα<sub>q</sub> protein via a conformational selection
mechanism. After a tilt of the helix A in the αH domain (Gαq helix A open → Gαq helix A closed), macrocyclic Gαq inhibitors can now bind to their binding site.

4. Discussion

The structurally similar cyclodepsipeptides FR and YM represent the only known non-protein entities inhibiting heterotrimeric G proteins with nanomolar potency, displaying selectivity for the Gαq/11/14 subunits [13,15,28,35]. Because of the high impact of these tool compounds on pharmacological research, detailed information about their binding properties and interactions with the target is essential. In the present study, we aimed at elucidating the details of the binding mechanism and at investigating the molecular determinants for the long residence time of the macrocyclic Gq protein inhibitor FR in comparison to its close analog YM. To this end, we expressed the wt and a series of mutant Gαq proteins in HEK293 Gαq/11-KO cells [13,62].

As previously observed in native platelets (24-fold difference in residence time at 37°C) [28], also in the recombinant system both radioligands showed a similarly large (20-fold) difference in dissociation kinetics and thus in residence time (1/k_{off}): 5.61 min for the YM-derived radioligand and 114 min for the FR-derived radioligand determined at 37°C (Fig. 3E).

Association of both compounds, however, was comparably fast (2.47 and 2.18 min, respectively) (Fig. 3D). In saturation binding assays, both radiotracers displayed nearly identical apparent pK_{D} values of around 8 (see Fig. 3B,C). These results were in agreement with data previously determined at platelet membrane preparations, and in accordance with pK_{D} values from pseudo-homologous competition binding studies (YM vs. ^{3}H]PSB-16254-YM, and FR vs. ^{3}H]PSB-15900-YM), in which both radioligands displayed virtually identical affinity at wt Gαq proteins expressed in HEK293 cells [28].

Whereas the calculated kinetic pK_{D} value of 9.5 obtained from association and dissociation experiments predicted a much higher affinity for [^{3}H]PSB-15900-FR, the kinetic pK_{D} value of [^{3}H]PSB-16254-YM was in good agreement with the apparent pK_{D} determined in the saturation
binding studies. However, both the calculation of the apparent pKₐ values from saturation binding and that of the kinetic pKₐ value assume a one-step binding model and do not account for possible conformational transitions in the protein during binding or unbinding of the ligand. By performing concentration-dependent kinetic measurements (see Fig. 3F,G), we now provide evidence for a more complex binding mechanism predominantly characterized by initial conformational selection. Both, a one-step binding model, and an induced-fit model would necessarily result in an increase in kₐ with increasing ligand concentration, [L] [44]. However, we observed an exponential decrease. Relevant kinetic parameters to describe a protein-ligand-interaction by a conformational selection model are the protein interconversion rates kᵣ and kᵣᵣ, which can be extracted from the Y-axis intercept (corresponding to the sum of kᵣ+kᵣᵣ) and the plateau (corresponding to kᵣ) of a plot of kₐ versus [L]. kᵣ describes the forward isomerization rate from a non-binding conformational ensemble of the protein towards a conformational ensemble that binds the ligand; conversely, kᵣᵣ describes the reverse isomerization rate. In the absence of a ligand, these protein states (labeled as Gα and Gα’ in Fig. 2) are at equilibrium, but with increasing concentrations of ligand (FR or YM), the concentration of free Gα decreases and the formation of Gα from Gα’ (kᵣ) becomes the rate-limiting step for ligand-protein complex formation. Analysis of the plots (Fig. 3F,G) yields similar interconversion rates of the wt Gαq protein with respect to both radioligands: forward isomerization of a non-binding conformation (Gα’) towards a conformation capable of binding [³H]PSB-15900-FR (Gα) occurs with a rate of kᵣ = 0.32 min⁻¹ (0.22 min⁻¹ for [³H]PSB-16254-YM). The respective reverse isomerization rate for the [³H]PSB-15900-FR-binding conformation is kᵣᵣ = 0.32 min⁻¹ (0.43 for [³H]PSB-16254-YM). A kinetic Kₐ value can be determined graphically from the plots as illustrated in Fig. 3 F,G, i.e. the concentration, at which kₐ equals the mean between the Y-axis intercept and the plateau. The kinetic Kₐ values determined by this method are 0.89 nM (pKₐ = 9.05) for [³H]PSB-15900-FR, and 3.86 nM (pKₐ = 8.41) for [³H]PSB-16254-YM, thus predicting a significantly higher affinity, especially for [³H]PSB-15900-FR, than the
determined apparent \( K_D \) values obtained by saturation experiments. These kinetic \( pK_D \) values are likely to be more accurate estimates of the compounds’ affinities, correlating better with the very different \( k_{off} \) values of both radioligands, and taking their complex binding mechanism into account. We conclude that forward isomerization is the rate-limiting step in the association of \(^{3}H\)PSB-15900-FR and \(^{3}H\)PSB-16254-YM to the wt \( G\alpha_q \) protein, which explains why determination of kinetic \( K_D \) values was not possible by equations designed for a one-step binding model.

Several reports on multi-step ligand binding to proteins have been published, either via induced fit or by conformational selection [45,63–65]. In the present study we show that the \( G\alpha_q \) protein inhibitors FR and YM bind through conformational selection. Taking into consideration the complex structure and the flexibility of the ligand, the major differences in residence time of both inhibitors, and the structural dynamics of \( G\alpha \) proteins [66–68], the entire binding mechanism may involve even further steps. Initial conformational selection is likely followed by induced-fit binding, possibly mediated by the hydrophobic anchors of FR, which act like “dowels” helping to retain the molecule in its binding pocket. Similar to a dowel or a snap-lock, FR and its radioligand are then presumably fixed by their lipophilic anchor residues, in particular by anchor 2. This mechanism explains the long residence time of FR in contrast to YM and its much higher kinetic \( pK_D \) value. Thus, the apparent \( pK_D \) value calculated for \(^{3}H\)PSB-15900-FR from saturation binding assays is markedly underestimated. It can be assumed that both inhibitors show an at least 4-fold difference in affinity as evidenced by their more accurate kinetic \( pK_D \) values derived from the two-step binding model.

In order to provide a structural explanation for the large differences in residence time, we mutated amino acid residues in \( G\alpha_q \) protein around anchors 1 and 2 of bound FR. These residues were chosen based on molecular modeling and docking studies (Fig. 1, mutations are visualized in Fig. 4); our rationale is detailed in Supp. Table 2. Only the F75A mutant could not be
expressed in our hands, while the F75K mutant lost high affinity binding and showed decreased
potency for both FR and YM (Fig. 5). F75<sup>H.HA.07</sup> forms part of a small cluster of
hydrophobic/aromatic amino acids (Y67<sup>G.h1ha.04</sup>, F75<sup>H.HA.07</sup>, V184<sup>G.hfs2.03</sup>) joining linker 1 and
switch 1, which link RasD and the αH domains of the Gα subunit. Our results show that
disruption of this cluster leads to severely impaired inhibitor binding.

Most of the designed Gα<sub>q</sub> mutants exhibited high specific binding of both radioligands with
similar affinity to the wt Gα<sub>q</sub> protein as determined in competition binding (Fig. 5B) and
calcium mobilization studies (Fig. 5C). However, the I190F and I190W mutations led to a loss
of high-affinity binding and reduced potency (Fig. 5, also see [69]). I190 interacts with adjacent
residues likely stabilizing switch 1; bulkier Phe and Trp side chains possibly perturb the local
structure of the binding site near anchor 1. Interestingly, the reduction in potency appeared to
be more pronounced for YM as compared to FR, especially in case of the I190W mutant (Fig.
5B) possibly because FR can compensate the unfavorable binding in the structurally altered
mutants by its tighter binding due to its lipophilic anchors.

However, major differences among the studied mutants were observed in the kinetic studies.
All mutations around anchors 1 and 2 dramatically accelerated the dissociation of the
radiotracers, especially that of the slowly dissociating radioligand [<sup>3</sup>H]PSB-15900-FR. This
result is complementary to the structure-activity relationships observed for analogs of YM and
FR [1,34,70] and a molecular networking analysis [71], which led to the conclusion that nearly
every part of the inhibitor molecule contributes to its pharmacophore. The Gα<sub>q</sub> protein
mutations accelerated the dissociation to variable extents. Kinetic data displayed the following
rank order regarding the impact of the Gα<sub>q</sub> protein mutations on the dissociation of [<sup>3</sup>H]PSB-
15900-FR: G74V > V184M > P193C > E191A, L78A > R202H.

The isopropyl residue (anchor 2) appears to play a key role for the long residence time of FR
(Fig. 6C) since mutations around this region had even larger negative effects on the residence
time of FR than those around anchor 1. The binding site near anchor 2 is likely tighter and
features hydrophobic interactions with nearby residues including F75$^{H.HA.07}$, L78$^{H.HA.10}$, and
V184$^{G.hfs2.03}$. Remarkably, the two most impactful single mutations analyzed are located around
anchor 2. The G74V and G74V/L78A mutants are of particular interest since they dramatically
reduced the dissociation half-life of [$^3$H]PSB-15900-FR by 88-fold and 198-fold, respectively,
whereas the already low dissociation half-life of [$^3$H]PSB-16254-YM was reduced by 5- and
13-fold, respectively, resulting in virtually identical, very short residence times for both
radioligands (0.53 and 0.43 min respectively). In the G74V mutant, the bulky side chain of the
valine group likely clashes with the isopropyl moiety (anchor 2) of [$^3$H]PSB-15900-FR,
resulting in a less stable binding pose with a faster off-rate. Conversely, exchange of L78$^{H.HA.10}$
for alanine removes a favorable hydrophobic interaction partner for anchor 2 leading to a
strongly reduced residence time of [$^3$H]PSB-15900-FR.

In comparison, the binding pocket near anchor 1 is wider, allowing for flexible movements of
the β-hydroxyleucine side chain of FR [28]. In this region, the only predicted interaction partner
for anchor 1 is P193$^{G.S02.05}$. The P193C and V184M mutants were inspired by the FR-insensitive
Ga$_{15/16}$ subunit, which is the closest Ga$_q$ paralog that does not bind the macrocyclic inhibitors
with high affinity. These Ga$_q$ mutants were previously found to be fully sensitive to YM and
FR in functional assays [33], which is in good agreement with our data from calcium assays.

However, we could now show that these single amino acid exchanges were sufficient to
accelerate radioligand dissociation significantly. Also around anchor 1, the E191A mutation
removes a salt bridge to the nearby R202$^{G.S03.04}$, which slowed down the association and
accelerated the dissociation of the radiotracers. However, the more conservative R202H
mutation, which maintains the positive charge, had no effect on association and a considerably
lower impact on dissociation kinetics. These results show that the salt bridge between
E191G.S02.03 and R202G.S03.04 (or H202 in the mutant) stabilizes a conformation of the β2/β3 sheet in the Gq protein that favors binding of the inhibitors.

Mutations around both anchors affected the dissociation half-life of the long-binding [3H]PSB-15900-FR to a far greater extent than that of the short-binding [3H]PSB-16254-YM (see Fig. 6 C + D and Supp. Table 6). For [3H]PSB-15900-FR, minor changes of the protein binding site caused unexpectedly large changes in its dissociation. Mutagenesis studies with a focus on the study of ligand binding kinetics are scarce; only a limited number of structure-kinetic relationship studies have been performed, e.g. for the β2-adrenoceptor [72], the cannabinoid CB2 receptor [73], and cyclic dependent kinase 8/cyclin C (CDK8/CycC) [74]. However, no commonalities have been established for long-lasting ligand-protein complexes so far, and the interactions required for long-duration binding appear to be case-specific. These may include deep-pocket binding (CDK8/CycC inhibitors) [74], or – like in the present study – lipophilic interactions [73]. Moreover, there have been reports on compounds displaying a similar equilibrium Ki value at their target receptor despite large differences in their koff values [75]. Residence time does not always correlate with the duration of action as shown e.g. for β2 adrenoceptor agonists [72], but in other cases, a correlation has been observed, e.g. for adenosine A2A receptor agonists [76]. In one of the few available in vivo studies of Gαq inhibitors, FR indeed inhibited metacholine-induced bronchoconstriction significantly longer than YM, and was pharmacologically active even 96 h after administration while the same dose of YM had lost all of its effect already after 48 h [17]. This report indicates that a long residence time leading to a long-lasting inhibition of Gαq is preferable, at least in the treatment of asthma bronchiale.

5. Conclusion

The previous development of radioligands obtained by 3H-labeling of the potent and selective macrocyclic Gαq protein inhibitors FR and YM allowed us to perform structure-kinetics
relationship studies at the Gα_q inhibitor binding site. Mutation of selected amino acids revealed key interactions responsible for the long residence time of FR as compared to YM. Some of these residues form hydrophobic interactions with the isopropyl anchor 2 of FR (residues L78^{H.A.10} and V184^{G.hfs2.03}), providing a molecular basis for its long residence time. Surprisingly, binding affinity of FR and YM, inhibitory potency, and association kinetics in most of the investigated Gα_q protein mutants appeared to be not significantly different to those of the wt Gα_q protein, while dissociation was found to be dramatically accelerated, especially for the FR-radioligand.

A detailed investigation of the binding mode revealed that the observed association rate $k_{obs}$ exponentially declines with increasing radioligand concentration. This strongly suggests that the radioligands bind to the wt Gα_q protein via conformational selection, which was supported by a molecular dynamics simulation study rationalizing the conformational changes and interactions. We suggest that the binding mechanism of macrocyclic Gα_q inhibitors, in particular that of the slowly dissociating FR, may involve yet further conformational changes, e.g., a “snap-lock”-like conformational rearrangement of the binding site around helix A in the αH domain of Gα_q partially mediated by the anchors of FR, which is disrupted in the mutants. This mechanism provides an explanation for the much slower dissociation of FR as compared to YM. Furthermore, our data clearly demonstrate that an in-depth investigation of binding kinetics is crucial for the characterization of protein-ligand interactions. The present data are of general importance since residence time is increasingly recognized as a crucial parameter in drug development.

Author contributions

JHV and JN conducted and analyzed pharmacological experiments; JHV, MR, JP, DM, and EK contributed to molecular biology; SK and GMK isolated, purified, analyzed, and provided FR900359; AI prepared and provided HEK293 Gα_q^{11}-KO cells; VN, RG, and XD performed...
and supervised the computational studies; VN, JHV, and CEM selected mutants; CEM conceived, coordinated and supervised the project; JHV and CEM wrote the manuscript with contributions from all coauthors.

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Declaration of conflicts of interest

All authors declare that they have no competing interests.

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