

Ligand channel in pharmacologically stabilized rhodopsin

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In the degenerative eye disease retinitis pigmentosa (RP), protein misfolding leads to fatal consequences for cell metabolism and rod and cone cell survival. To stop disease progression, a therapeutic approach focuses on stabilizing inherited protein mutants of the G protein-coupled receptor (GPCR) rhodopsin using pharmacological chaperones (PC) that improve receptor folding and trafficking. In this study, we discovered stabilizing nonretinal small molecules by virtual and thermofluor screening and determined the crystal structure of pharmacologically stabilized opsin at 2.4 Å resolution using one of the stabilizing hits (S-RS1). Chemical modification of S-RS1 and further structural analysis revealed the core binding motif of this class of rhodopsin stabilizers bound at the orthosteric binding site. Furthermore, previously unobserved conformational changes are visible at the intradiscal side of the seven-transmembrane helix bundle. A hallmark of this conformation is an open channel connecting the ligand binding site with the membrane and the intradiscal lumen of rod outer segments. Sufficient in size, the passage permits the exchange of hydrophobic ligands such as retinal. The results broaden our understanding of rhodopsin's conformational flexibility and enable therapeutic drug intervention against rhodopsin-related retinitis pigmentosa.

structural biology | drug discovery | chemical biology | rare diseases | ophthalmology

Retinitis pigmentosa (RP) unites a class of hereditary diseases causing the progressive degeneration of the photoreceptor system (1) and visual impairment in 1.5 million patients worldwide (2). Predominantly affected are rod cells in the retina of the eye that aggregate and progressively affect cone cell viability (3). One reason is the inherited RP mutation P23H in rhodopsin discovered nearly 30 y ago as one of the first examples for a disease-related G protein-coupled receptor (GPCR) mutation (4). Over 100 more followed over the years, sparking the idea to treat this common cause of age-related blindness by directly targeting the mutated photoreceptor opsin (5, 6). In contrast to most GPCR drugs that modulate signaling, drugs acting on opsin mutants have to intervene early in protein synthesis and folding to compensate for the observed decrease in protein stability (7) and for the fatal consequences for cell metabolism and rod and cone cell survival (3).

A pharmacological chaperone (PC) treatment of rhodopsin-mediated RP can stabilize and increase opsin resistance to facilitate correct transport to the disk membranes of the rod outer segment (ROS). This is in analogy to the effect of known orthosteric and allosteric ligands that promote expression and correct protein folding and trafficking for a number of GPCRs (8, 9). In a mouse model of failed puberty, PC therapy could restore the fertility of mice by correctly routing the mutated gonadotropin-releasing hormone receptor to the plasma membrane (10). Notably, a very small subset of organic molecules was able to address most of the mutants causing the disease phenotype (10). Similarly, the endogenous ligand 11-*cis* retinal assists folding and integration of opsin RP mutants in vitro and in vivo (7, 11–14). However, the toxicity of 11-*cis*

retinal and its regulated transport to and from the ROS limits its potential as pharmacological chaperone (15, 16). Here, we screened for nonretinoid drug-like rhodopsin stabilizers and studied their molecular interactions with the photoreceptor protein by X-ray crystallography. Comparing our pharmacologically stabilized structures of opsin to existing active- and inactive-state rhodopsin structures, we identify distinctive conformational rearrangements and an open ligand channel to the intradiscal side.

Results and Discussion

Nonretinal Opsin-Stabilizing Compounds. In congenital stationary night blindness, the mild and nonprogressive phenotype of RP, mutations lead to increased basal activity by selectively stabilizing the orthosteric binding site (17, 18). In contrast, severe RP mutants have a destabilizing effect on rhodopsin protein (5, 6). To counterbalance, pharmacological rescue aims at intervening early in protein genesis to improve folding and trafficking in vivo (14) and in vitro (7, 11–13), as demonstrated so far only for retinoid-like molecules (16). Binding and stabilization of mutated receptor protein initiates the

Significance

A substantial number of known genetic disorders have their origin in mutations that cause misfolding or dysfunction of G protein-coupled receptors (GPCRs). Pharmacological chaperones can rescue such mutant receptors from the endoplasmic reticulum by stabilizing protein conformations that support trafficking into the target membrane. Rhodopsin-mediated retinitis pigmentosa is a misfolding disease that might be targeted by PCs. Here we present a structure-based drug design approach to identify non-retinal compounds that bind and stabilize the receptor. Surprisingly, selected hits induce a previously unknown conformation of the seven-transmembrane helix bundle. Our study thus provides a remarkable example for compound class discovery and for the adaptability of GPCRs to chemically diverse ligands.

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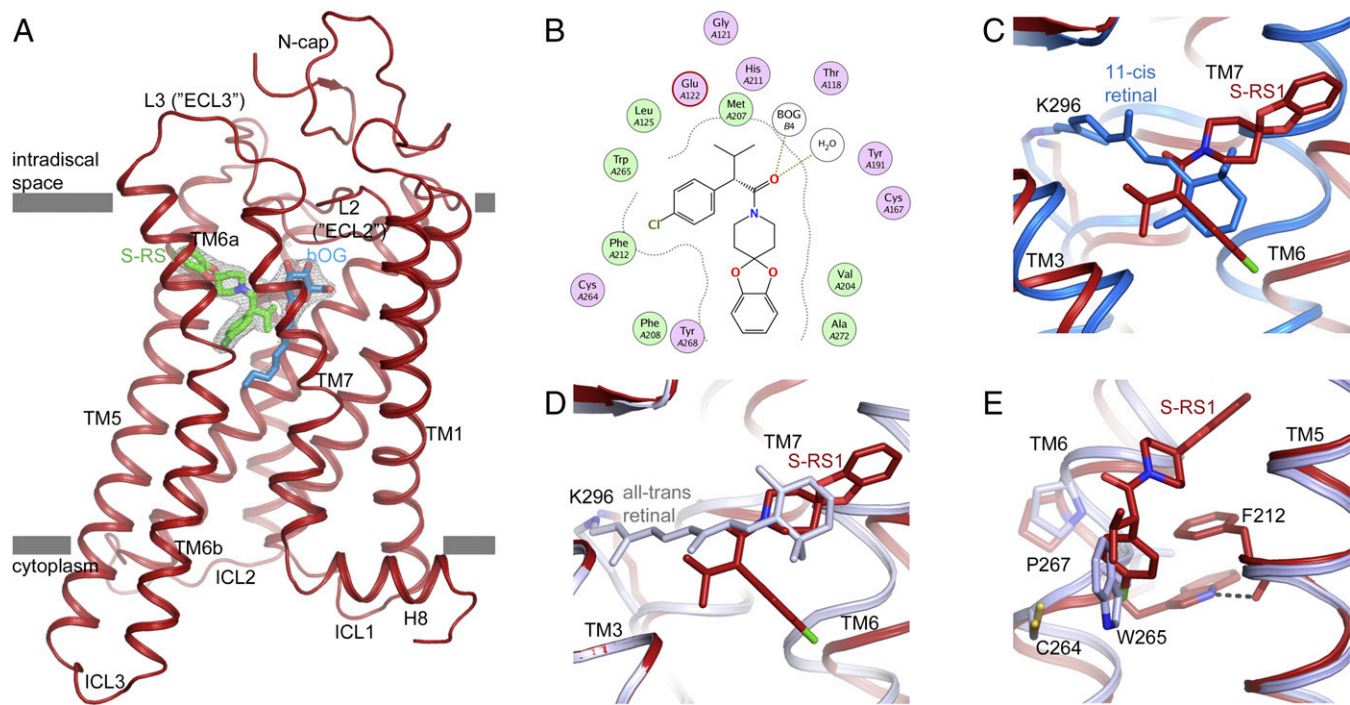
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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.rcsb.org [PDB ID codes 6FK6 (S-RS01), 6FK7 (RS06), 6FK8 (RS08), 6FK9 (RS09), 6FKA (RS11), 6FKB (RS13), 6FKC (RS15) and 6FKD (RS16)]. Additional data related to this publication are available at the Cambridge Structural Database, <https://www.ccdc.cam.ac.uk/solutions/csd-system/components/csd/> [CCDC codes 1819787 (R-RS1) and 1819788 (S-RS1)].

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Of particular interest is the close interaction between the isopropyl group in position C21 of S-RS1 and opsin's carboxyl group of Glu122^{3,37} in TM3. This location, together with the adjacent Gly121^{3,36}, plays a role in retinal selectivity (26), and S-, M-, and

L-cone opsins feature distinct amino acids at this position. Opsin exhibits stereoselectivity for the S enantiomer of the chiral compound S-RS1 likely due to steric hindrances for the R enantiomer. Structure refinement using the R enantiomer R-RS1 resulted in structural

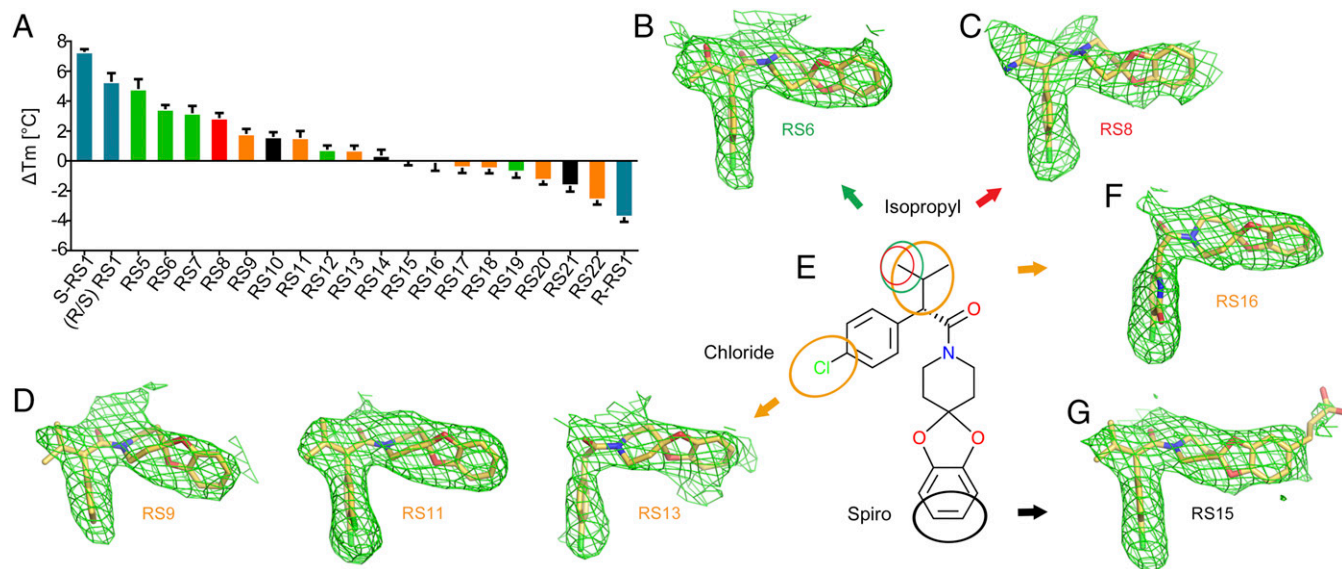


Fig. 3. Crystal structures and thermofluor analysis of S-RS1 derivatives. (A) Experimentally determined ΔT_m values for RS1 stereoisomers (blue) and derivatives grouped by alterations at the isopropyl and chlorine (orange), isopropyl (green, red), or spiro function (black). (B–G) Side view of the ligand binding site of opsin (model not shown) in complex with compounds in stick representation. The compound color code reflects each group for limited chemical modification. Experimental electron density maps ($F_o - F_c$) in green mesh are contoured at 3σ . (E) Chemical structure of S-RS1.

clashes. Dose-response thermofluor analysis of both S and R configurations of RS1 revealed that no stabilization could be measured for the R enantiomer (Fig. 1*B* and *SI Appendix*, Fig. S6). Modification of S-RS1 with an amino group at position C24 or a hydroxyl group at position C25 was neutral for stabilization yet resulted in crystal structures and modified hydrophilic groups in H-bonding distance to Glu122^{3,37}. Modifications impacting position C21 prevented cocrystallization.

The chlorine of the S-RS1 phenyl chlorine ring forms interactions with two crucial binding pocket residues, namely, the sulfhydryl group of Cys264^{2,47} and several atoms of Trp265^{2,48}. Removal of the chlorine as well as addition of an extra chlorine in position C23 diminished stabilization although we observed only minimal changes in the binding mode. Larger modification at these positions and of the ring size abolished binding completely. This renders the region critical for binding similar to the position C21 isopropyl group limiting S-RS1 modification for improved solubility and permeability.

We speculated about the spiro ring modification of S-RS1 to improve solubility and affinity, since an initial hit of thermofluor screening and precursor of S-RS1 did not contain this ring function (Fig. 1*A*). To probe the large cavity, we added a carboxyl group connected with a linker to C16 of the S-RS1 spiro ring resulting in additional interactions with Tyr191^{ECL2} and Met288^{7,35}, while a rotamer change buries Phe276^{6,59} into the hydrophobic environment surrounding the receptor. Both thermofluor and FSEC-TS analysis revealed maximal stabilization among the S-RS1 derivatives. This side of the binding pocket may thus be a location to further probe the chemical space and improve on the initial rhodopsin stabilizers. The methods we used in our approach, including virtual screening, thermal shift analysis, retinal competition, cellular trafficking, and structural analysis, are very robust and suggest good applicability for drug discovery. Furthermore, the results indicate that rhodopsin is a

tractable target for structure-based drug design and that the early compounds described in this study can be further optimized.

A Ligand Channel Toward the Extracellular Side. Ligand-induced conformational changes are a hallmark of GPCR activation. In rhodopsin, translocation of retinal's β -ionone ring is a critical step during receptor activation. It allows TM6 to rotate into the active conformation and to provide access to the G protein binding site (27–29). Interestingly, structure comparison reveals that the positions of all stabilizing S-RS1-based compounds simultaneously overlap with the positions of retinal's β -ionone ring in the dark and active meta-II states. In other words, the angular shape of S-RS1 stabilizes opsin simultaneously at locations of the orthosteric binding pocket known for the agonist all-*trans* retinal or the inverse agonist 11-*cis* retinal. The chlorinated phenyl ring is located at the position of the β -ionone ring in the dark state, and the spiro group overlaps with the position in the meta-II conformation (Fig. 2*C* and *D* and *SI Appendix*, Fig. S7). Occupation of these positions by the compound forces Trp265^{6,48} of the highly conserved CWxP sequence motif found in class-A GPCRs to rotate into a new rotamer position outside the 7TM bundle (Fig. 2*E*). The outward position is maintained by a hydrogen bond with the backbone carbonyl of Phe212^{5,47} and π -stacking with the phenyl group of Phe212^{5,47} in TM5. This dislocation of Trp265^{6,48} toward a more polar environment was not reported in previous crystal structures of rhodopsin or other class-A GPCRs, but is in good agreement with predictions from linear dichroism (30) and UV/VIS spectroscopy (31–33). Importantly, investigation of rhodopsin decay kinetics by NMR spectroscopy with ¹⁵N-labeled tryptophan further supports the view of a position of Trp265^{6,48} during rhodopsin decay that is different from both positions in the dark and meta-II states (34).

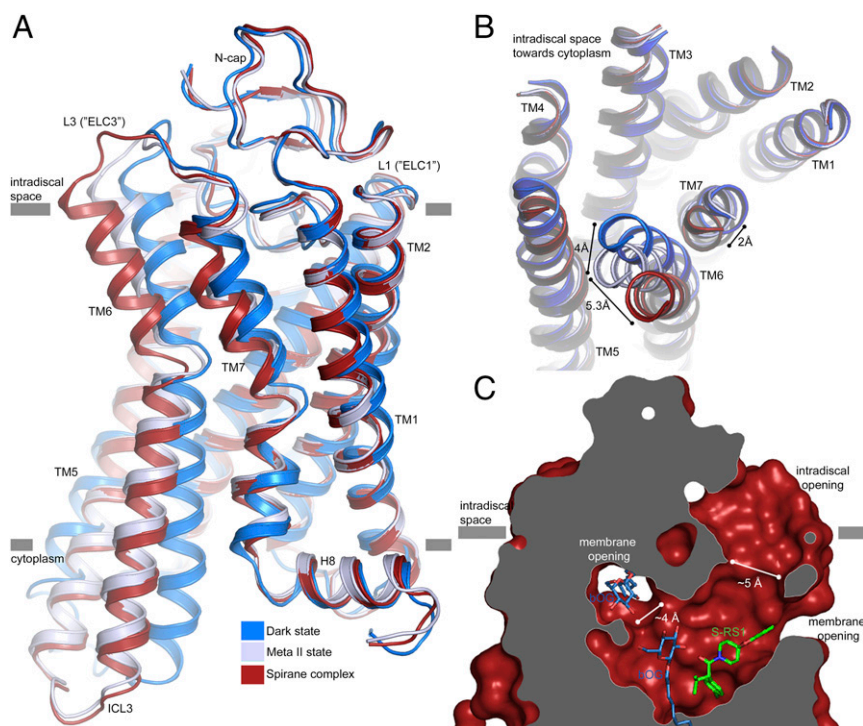


Fig. 4. A ligand channel in S-RS1-stabilized rhodopsin. (A) Side view and (B) top view onto the superposition of the S-RS1-bound conformation (red) of opsin-, dark- (PDB ID: 1GZM, blue), and meta-II-state rhodopsin (PDB ID: 3PQR, blue-white) visualizing the helical movements in TM6 and TM7 at the intradiscal side of the membrane. The complex has a higher structural similarity to meta-II (rmsd = 0.4 Å) than dark-state rhodopsin (rmsd = 1.6 Å). (C) Ligand channel in surface representation sliced perpendicular to the membrane highlighting an intradiscal opening formed between L2 ("ECL2"), TM5, and TM6a as well as a membrane opening, large enough to permit the exchange of hydrophobic ligands including retinal.

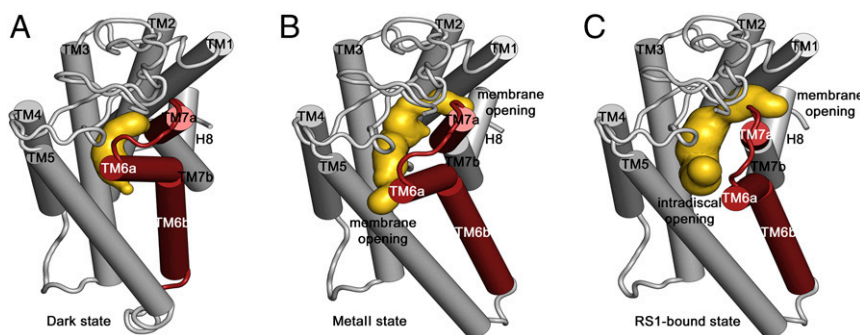


Fig. 5. Ligand channel comparison of dark, meta-II, and S-RS1-bound opsin. Cartoon representation of the receptor depicting unaltered transmembrane helix regions (gray) and region of ligand-induced conformational changes (red). The caver analysis reveals the void pathways of the receptor in yellow of (A) dark-state rhodopsin bound to 11-*cis* retinal featuring an occluded pocket, (B) light-activated meta-II rhodopsin with bound all-*trans* retinal showing two small openings toward the membrane, and (C) S-RS1-bound opsin identifying a ligand channel toward the membrane and intradiscal space suggesting a potential route for retinal exchange and ligand-induced receptor breathing.

On the intradiscal side, repositioning of Trp265^{2,48} is accompanied by a significant rearrangement of the seven transmembrane helix bundle (Fig. 4A). In particular, TM6 shifts by 4 and 5.3 Å in comparison with the meta-II and dark states, respectively (Fig. 4B). Smaller adjustments are visible for TM7 as well as for intradiscal loops L2 and L3 that correspond to the extracellular loops ECL2 and ECL3 of other GPCRs. This conformational reorganization creates a large opening, defined by residues of TM5, TM6, L2, and L3 that connect the orthosteric binding site with the lipid bilayer and the lumen of the rod outer segment disk (Fig. 4C and *SI Appendix*, Fig. S8). Residues exposed at the extracellular boundary are primarily hydrophobic and highly conserved (Phe208^{3,43}, Phe212^{5,47}, Tyr268^{6,51}, Val204^{5,39}, Phe276^{6,59}, Ala272^{6,55}, Phe283, Tyr191, Tyr192, and Met288^{7,35}) (*SI Appendix*, Fig. S9). We also observe a second cavity located between TM1 and TM7 pointing toward the cytosolic leaflet of the membrane. The boundary is primarily lined by hydrophobic residues of low conservation (Phe293^{7,40}, Ile286^{7,33}, Met39^{1,34}, Leu40^{1,35}, and Met183) similar to the ones reported earlier (*SI Appendix*, Fig. S9) (35). This second cavity is much smaller but large enough in size to harbor a detergent molecule whose head group is buried inside, the alkyl chain pointing toward the membrane (*SI Appendix*, Fig. S4).

In the closely related β -adrenergic receptors, a channel through the extracellular domain allows entry and exit of the diffusible ligands needed for activation (36, 37). In the case of dark-state rhodopsin, this channel is not observed (Fig. 5A). On the other hand, retinal was proposed to enter and exit to the lipidic membrane through two openings located between TMs 1 and 7 and TMs 5 and 6 in the near-identical active opsin and meta-II conformations (Fig. 5B) (20, 35), yet an extensive mutational analysis of this channel remained inconclusive (38). Recent studies established that retinals bind selectively to different conformations with 11-*cis* retinal entering through a putative new open conformation not seen in previous crystal structures (39, 40). The diameter of the intradiscal channel in our structure is between 4.8 Å and 6.8 Å in diameter, i.e., wide enough to allow passage of retinals (Fig. 5C), and is in good agreement with mutational data for binding and release of retinal (38). It is thus tempting to speculate that the surprisingly open conformation of the S-RS1-opsin complex (Fig. 5C) may represent a physiologically relevant transition state by which retinals can enter or leave the orthosteric binding site.

Conclusion

One of the greatest challenges to make use of structure–activity relationships in GPCRs is their conformational flexibility. Atomic resolution information provides a practical entry point for rational drug design. Pharmacologically stabilized opsin is a reminder of how

unnatural ligands can stabilize previously unresolved protein conformations, even in a tightly regulated GPCR such as rhodopsin.

Our study clearly demonstrates that rhodopsin is a pharmacological target similar to other GPCRs and remains a valuable system to study GPCRs. The S-RS1 compound and derivatives are particularly interesting because their angular shape simultaneously probes the orthosteric binding pocket similar to how the agonistic or inverse agonistic endogenous ligand do individually. A next generation of pharmacological chaperones will thus not only stabilize a particular conformation but will likely support different stages of the folding pathway providing a valuable framework for the pharmacological rescue of rhodopsin-mediated retinitis pigmentosa. Reversible binding will further allow 11-*cis* retinal to form a stable pigment once stabilized opsin reaches the rod outer segment membrane.

Materials and Methods

In Silico Selection of Focused Library. Three different types of 2D ligand- and 3D shape-based similarity calculations using all-*trans* retinal and three published retinoid opsin binders (41, 42) as references, as well as two structure-based methods, namely, pharmacophore searching and docking, were used to compile the focused library. The crystal structure of rhodopsin with bound all-*trans* retinal [PDB ID: 4A4M (20)] was used as a template for a pharmacophore model performed in the software program MOE (Molecular Operating Environment software package; available at https://www.chemcomp.com/MOE-Molecular_Operating_Environment.htm) including five hydrophobic features covering the β -ionone ring and the region close to the ligand head group, as well as excluded volume features for the surrounding protein except for the flexible, retinal-binding Lys296^{7,43}. Additional pharmacophore searches were performed with models mimicking a potential interaction with Lys296^{7,43}. Molecular docking was performed with an additional hydrogen-bonding constraint between any ligand atom and NZ of Lys296^{7,43} to exclude covalent binders.

Protein Production, Crystal Soaking, and Structure Determination. A detailed description of the cloning, expression, purification, and crystallization process of human opsin N2C/N282C and bovine opsin N2C/D282C can be found here (29, 43, 44). Crystal soaks using 3.75–5 mM compound concentrations were incubated for 24 h before flash-cooling of crystals with liquid nitrogen. Statistics of structure determination are listed in *SI Appendix*, Table S1.

Automated Thermofluor Assay. A published assay (45) based on the *N*-(4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl)maleimide (cpm) dye was adapted and automated to screen for stabilizing compounds. Melting curves were obtained applying a temperature gradient from 25 to 95 °C and a heating rate of 0.25 °C/s. All liquid-handling steps were performed using a Bravo Automated Liquid handling platform and a 96LT head. Melting temperatures (T_m) were calculated and analyzed by automated scripts based on a fit to the Boltzmann equation.

Fluorescence-Detection Size-Exclusion Chromatography Thermal Shift Assay.

The method was used to validate the compound-induced stabilization of human opsin N2C/N282C (46). Melting temperatures were determined in duplicate by incubation of 0.26 μ M human opsin N2C N282C with and without compounds at 10 μ M concentration (10 mM stock solution in DMSO) for 10 min in a Prime Thermal cycler at 12 different temperatures between 35 and 60 °C in an Eppendorf twin.tec PCR plate in a buffer containing 10 mM Hepes (HCl) pH 7.4, 200 mM NaCl, 0.125% (wt/vol) *n*-decyl- β -D-maltopyranoside. Nonaggregated protein was quantified using tryptophan fluorescence after injection of 4 μ L protein solution and separation on an HPLC system ($\lambda_{\text{excitation}}$ of 280 nm, $\lambda_{\text{emission}}$ of 350 nm). Melting temperatures (T_m) were determined using the Boltzmann equation fit in GraphPad Prism including normalization defining 0% as the smallest and 100% as the largest identified value within the tryptophan fluorescence dataset.

Pharmacotraficking Complementation Assay. Commercial rhodopsin P23H stable cell lines (ID 93–1077C3; DiscoverRx) were used to express a C-terminal fusion of mouse P23H opsin with the small β -galactosidase subunit and the fusion of the large subunit of β -galactosidase and the pleckstrin homology domain of phospholipase C- δ as described (22). For measurements, the cell medium was exchanged to reaction reagent, and ~5,000 cells per reaction were transferred to a 384-well plate. Detection of cell surface expression was performed with PathHunter Detection Kit reagent in a luminescence plate reader. Measurements were evaluated with GraphPad Prism using the normalized dose-response curve with the lowest value being defined as 0% and the highest value as 100% in the compound dataset. All experiments were

done under red-light conditions due to the instability of the control 9-*cis* retinal to normal light.

Compound Production and Characterization. S- and R-RS1, the racemate, and additional compounds were synthesized at Chembiotek and purified. All reactions were monitored by TLC, and the reaction construct was confirmed by NMR. The purity of final compounds as measured by liquid chromatography coupled to mass spectrometry was at least >95%. The two enantiomers of RS1 were separated on a Reprosil Chiral NR column. The identity of compounds S-RS1 and R-RS1 was further confirmed by small-molecule crystallography (SI Appendix, Table S4).

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