A High-Throughput Screening Method for Small-Molecule Pharmacologic Chaperones of Misfolded Rhodopsin

Syed M. Noorwez,1 David A. Ostrov,2 J. Hugh McDowell,1 Mark P. Krebs,1 and Shalesh Kaushal1,5

PURPOSE. Many mutations in rhodopsin, including P23H, result in misfolding and mislocalization of the protein. It has been demonstrated that pharmacologic chaperones are effective in assisting the proper folding and targeting of P23H opsin. This study was designed to investigate a high-throughput screening strategy for identification of pharmacologic chaperones by using a combination of in silico, cell-based, and in vitro methods.

METHODS. A library of 24,000 drug-like small molecules was screened by in silico molecular docking with DOCK5.1. The top hits were assayed in an in vitro competition assay. The selected compound was then assayed for pharmacologic chaperoning activity in stable cell lines expressing wild-type and P23H opsin.

RESULTS. β-Ionone was easily identified by the high-throughput screen. It strongly inhibits rhodopsin formation and, when incubated in cells expressing P23H opsin, resulted in a 2.5-fold rescue of P23H opsin. The screen also identified compound NSC45012 [1-(3,5-dimethyl-1H-pyrazol-4-yl)ethanone], a weak inhibitor of opsin regeneration and resulted in a 40% rescue of the mutant opsin. The level of rescue correlated well with the extent of inhibition.

CONCLUSIONS. A combination of in silico and cell-based screening provides a useful tool for identifying pharmacologic chaperones for P23H opsin. This approach identified both potent and weak pharmacologic chaperones. Both types of molecules may be potential candidates for treatment of opsin-related RP.

Small-molecule–based strategies have been intensely investigated in recent years as potential treatments for protein conformational diseases (PCDs).1,2,16,17 These diseases arise from mutations that result in their mislocalization or retention by the cellular quality-control machinery.3,4 Examples of PCDs include systemic diseases, such as cystic fibrosis, and progressive neuropathies, such as Alzheimer’s disease, Parkinson’s disease, and some forms of retinitis pigmentosa (RP).3,5,6 Many small molecules act as pharmacologic chaperones that bind and stabilize mutant proteins, allowing their release from the quality-control apparatus of the cell, potentially arresting or reversing diseases.7,8 Native ligands, substrate analogues, and enzyme inhibitors have been identified as pharmacologic chaperones for many misfolded proteins, including G-protein-coupled receptors (GPCRs).3,4,9,10

RP comprises a heterogeneous group of inherited retinal disorders that lead to photoreceptor death, resulting in night blindness and loss of central vision.9,12 Nearly 20% to 25% of patients with autosomal dominant RP have a mutation in rhodopsin. The P23H mutation accounts for approximately one third of such cases in the United States. P23H opsin forms only small amounts of pigment with retinal and most of the opsin is retained within cells as aggregates,13,14 providing a good system to study PCDs.9,16,17

Several lines of evidence suggest that pharmacologic chaperones may be effective in treating RP caused by mutant rhodopsin. In mammalian tissue culture systems, retinoids act as pharmacologic chaperones for opsin.9,14,16,17 These compounds are known to interact directly with the retinal-binding site of the opsin. Despite significant information available on the mechanism of inhibition, it is not known whether they can also pharmacologically rescue misfolded mutant opsins. Since pharmacologic chaperoning requires a compound to interact specifically with the target protein, these inhibitors may potentially rescue the mutant opsins. In the present study, we demonstrate that inhibitors of pigment formation act as pharmacologic chaperones and that there is a correlation between the potency of inhibition and rescue.

There is a need for a sensitive high-throughput assay method for screening potential pharmacologic chaperones that identifies compounds with low affinities for rhodopsin. Such an assay would lead to identification of a library of molecules with variable affinities that can be further assayed for activity. In recent years, many high-throughput methodologies have been developed to identify activators and inhibitors of many proteins,22,23 In silico computational methods that use the parameters of the ligand-binding sites of proteins have also been increasingly used to identify potential interacting molecules. They provide advantages over conventional screening strategies, which are laborious and time consuming.24 Better docking algorithms and multiprocessor resources have improved the technique of molecular docking to the point that it can be applied to more challenging problems.

In this study, we present an efficient combinatorial approach involving in silico, cell-based, and in vitro assays that is capable of identifying pharmacologic chaperones of misfolded P23H opsin.
After 48 hours of induction, WT opsin-producing cells were washed in PBS (10 mM sodium phosphate and 130 mM NaCl [pH 7.2]) and incubated for 48 hours.

**Figure 1.** Chemical structures of 11-cis retinal and its competitors. (A) Retinal has a bent conformation and contains an aldehyde group at the end. (B) Structure of \( \beta \)-ionone, which shares the 6-carbon ring and methyl groups at positions 1 and 5 of the ring with retinal. It contains a ketone group at the end. (C) Structure of \( \text{cis}\text{-}1,3\text{-dimethylcyclohexane} \).

**Materials and Methods**

**Database Preparation**

The National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) maintains a repository of approximately 240,000 small molecules (MW <500; the plated compound set). The three-dimensional coordinates for the NCI/DTP plated compound set was obtained in the MDL SD format and converted to the mol2 format by the DOCK utility program SF2MOL2 (hosted in the public domain by the University of California San Francisco, dock.compbio.ucsf.edu/dock_6/index.htm). Partial atomic charges, solvation energies, and van der Waals parameters for the ligands were calculated (SYBDB; Tripos, Inc., St. Louis, MO) and added to the plated compound set mol2 file.

**Molecular Docking**

All docking calculations were performed with the October 15, 2002, development version of DOCK, ver. 5.1.0.25 The general features of DOCK include rigid orienting of ligands to receptor spheres, AMBER (Assisted Model Building with Energy Refinement) energy scoring, GB/SA (Generalized-Born Model of Solvation for Small Molecules) solvation scoring, contact scoring, internal nonbonded energy scoring, ligand flexibility, and both rigid and torsional simplex minimization. Unlike previously distributed versions, this release incorporates automated matching, internal energy (used in flexible docking), scoring function hierarchy, and new minimizer termination criteria. The coordinates for the crystal structure of rhodopsin, PDB code 1GZM, were generated according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The result-ant plasmid allows site-specific recombination at the unique chromosomal FRT site of HEK293 Flp-In T-Rex. The stable cell lines were generated according to the manufacturer’s protocol (Invitrogen). Briefly, HEK293 Flp-In T-Rex cells were cotransfected with the flippase vector pOG44 and the pcDNA5/FRT/TO vector. The stable recombinants were obtained by selecting for cells expressing resistance to hygromycin. The stable cells were grown in DMEM with 10% fetal bovine serum, 5 µg/mL blasticidin, and hygromycin at 37°C and 8% CO_2. Cells were induced with 1 µg/mL tetracycline before addition of compounds and incubated for 48 hours.

**Plasmid and Stable Cell Line Construction**

To create plasmids for constructing stable cell lines, we introduced the wild-type (WT) or P23H mutant synthetic bovine opsin gene in pMT413 in the pcDNA5/FRT/TO vector (Invitrogen, Carlsbad, CA). The resultant plasmid allows site-specific recombination at the unique chromosomal FRT site of HEK293 Flp-In T-Rex. The stable cell lines were generated according to the manufacturer’s protocol (Invitrogen). Briefly, HEK293 Flp-In T-Rex cells were cotransfected with the flippase vector pOG44 and the pcDNA5/FRT/TO vector. Stable recombinants were obtained by selecting for cells expressing resistance to hygromycin. The stable cells were grown in DMEM with 10% fetal bovine serum, 5 µg/mL blasticidin, and hygromycin at 37°C and 8% CO_2. Cells were induced with 1 µg/mL tetracycline before addition of compounds and incubated for 48 hours.

**Opsi Purification, Regeneration, and 11-cis Retinal Competition**

After 48 hours of induction, WT opsin-producing cells were washed with PBS (10 mM sodium phosphate and 130 mM NaCl [pH 7.2]) and lysed in PBS (PBS containing 1.0% DM and 1× protease inhibitor cocktail) for 1 hour. The lysate was added to 1D4-coupled Sepharose beads and incubated for 1 hour at 4°C. WT opsin was eluted with a competing peptide corresponding to the last 18 amino acids of rhodopsin in the same buffer. The purified opsin was immediately used for regeneration and competition studies with selected NCI compounds and \( \beta \)-ionone.

The opsin regeneration and competition assays were performed in a spectrophotometer (Cary 50; Varian, Palo Alto, CA) equipped with temperature control. Purified WT opsin (25 µM) was mixed with 50 µM 11-cis retinal and scanned every 2 minutes in the range of 250 to 650 nm until no more rhodopsin was regenerated. Similarly, opsin was mixed with the compound NSC45012 (2 or 5 mM from 100 and 500 mM DMSO stock, respectively) and allowed to sit for 10 minutes on ice. Then, the retinal was mixed and spectra recorded every 2 minutes. \( \beta \)-ionone was added at 5- and 50-µM concentrations (from 500-µM and 2.5-mM ethanolic stock solutions). The temperature was maintained at 20°C.

**Cell-Based Screening of Compounds and Rhodopsin Purification**

For the study of total opsin, DMSO solutions of the NCI compounds and \( \beta \)-ionone were individually added to P23H cells at a final concentration of 100 and 10 µM, respectively. After incubation cells were lysed in PBSD and total protein was quantified with a DC protein assay (Bio-Rad). Equal amounts of protein (10 µg) were loaded on 4% to 20% SDS polyacrylamide gels, and total opsin was quantified by Western blot analysis with an infrared scanner and software (Odyssey; Li-Cor, Lincoln, NE).

For purification of rhodopsin, 100 µM NSC45012 or 10 µM \( \beta \)-ionone was added to the P23H cells. Rhodopsin purification was essentially as described earlier for opsin purification after incubation of cells with 50 µM retinal.

**Results**

**Pharmacologic Chaperoning by Inhibitors of Opisin Regeneration**

Small molecules that bind to the retinal binding pocket of opsin are predicted to stabilize the mutant opsin and to act as pharmacologic chaperones. To test this prediction, we studied known inhibitors of retinal binding in vitro and their pharmacologic chaperone activity in living cells. Several studies have identified compounds that compete with retinal, which consists of a six-membered cyclical head and a polyene chain with six alternating single and double bonds (Fig. 1A), for binding to opsin based on formation of rhodopsin with its characteristic absorption maximum at 500 nm. The methyl groups at positions 1 and 5 of the ring have been shown to be essential for anchoring of the retinal in the binding site.20 From these compounds, we selected \( \beta \)-ionone (Fig. 1B), which has a six-membered ring configuration like retinal but with a shorter side chain.19,21 It effectively competes with retinal for the chromophore-binding site. Like retinal, the six carbon ring of
β-ionone also has methyl groups at positions 1 and 5, but unlike retinal, it carries a ketone group at the terminal end.\textsuperscript{18,19,21,27} In addition, we selected cis-1,3-dimethylcyclohexane, which shares the six carbon ring with retinal and β-ionone and bears methyl groups at similar positions, but lacks a polycene chain (Fig. 1C). It is a significantly weaker inhibitor of opsin regeneration.\textsuperscript{27}

To examine the relative efficiency of small molecules at binding opsin, we first validated an in vitro assay, in which heterologously expressed purified wild-type (WT) opsin in detergent micelles was combined with retinal (Fig. 2A) in the absence or presence of compounds. In our in vitro experimental conditions, β-ionone inhibited WT opsin regeneration with retinal in a dose-dependent manner, thus demonstrating the competitive nature of the interaction (Fig. 2B). In the absence of β-ionone, the pigment formation plateaued in 30 minutes. In the same period, only 85\% or 40\% pigment was generated in the presence of 5 or 20 μM β-ionone, respectively. As reported previously,\textsuperscript{20,21} no 500-nm absorbing pigment was formed on addition of β-ionone to purified WT opsin (data not shown). These results provide evidence that β-ionone competed with retinal for the binding site.

To test whether β-ionone could serve as a pharmacologic chaperone, we added it to HEK293 stable cells expressing P23H opsin. Cells were harvested after 48 hours and then incubated with retinal. For comparison, retinal was added to separate P23H cells at the time of opsin induction. Rhodopsin was then purified under conditions that selectively yield the folded protein. Treatment with β-ionone led to a 2.5-fold increase in P23H rhodopsin (Fig. 2C, long-dashed line) over the control levels (Fig. 2C, solid line). The presence of retinal led to a fivefold increase in pigment yield, consistent with previous findings\textsuperscript{17} (Fig. 2C short dashed line). To study whether the increase in folded rhodopsin is due to increased accumulation of the protein, or simply because of an increase in the folded fraction, we measured total opsin by quantitative Western blot analysis. Total opsin levels increased 2.2-fold with β-ionone (Fig. 2D) and 5.6-fold in the presence of retinal. To rule out the possibility that a 500-nm pigment is formed by a β-ionone metabolite, P23H opsin was purified from cells treated with β-ionone alone, and it revealed that no 500-nm absorbing pigment was formed (Fig. 2E, solid line). Pigment was observed only after treating the cells with retinal (Fig. 2E, long dashed long). Similarly, to study the rescuing potential of the weaker inhibitor cis-1,3-dimethylcyclohexane (Fig. 1C), we added it to cells expressing P23H opsin. Quantitative purification showed that only a 1.2-fold increase in rhodopsin yield was achieved (Fig. 2E), which is consistent with its being a weaker inhibitor.

Collectively, the β-ionone and cis-1,3-dimethylcyclohexane results suggest that these compounds, which are structurally related to retinal, compete with retinal binding in vitro and are good candidate pharmacologic chaperones.

**Molecular Docking Studies to Identify Nonretinoid Compounds that Stabilize the Retinal Binding Pocket of Opsi**

The results just reported demonstrated that binding of a compound to the retinal-binding pocket can be a good predictor of its pharmacologic chaperoning capability. As demonstrated, assaying the inhibitory potential of a small set of molecules structurally related to the retinal is relatively easy when using an in vitro competition assay. But screening a large number of nonretinoids would be a challenging task. Therefore, to iden-
tify nonretinoids that might interact with the binding site of opsin, we used the in silico method. We used the x-ray crystallographic structure of WT bovine rhodopsin as a basis for molecular docking. We selected the site for molecular docking based on the position of retinal bound to rhodopsin (shown as yellow spheres in Fig. 3A). A scoring grid base to encompass the region was calculated around the selected site. A chemical library of 20,000 drug-like small-molecules from the NCI repository was selected for screening by molecular docking. Unlike previous molecular docking strategies, each of the docked compounds was preselected based on the Lipinski rules for drug likeness. These physical and chemical characteristics define orally deliverable drugs based on absorption and tissue permeability. Each compound was positioned in 100 different orientations by using DOCK5.1. We selected the top five scoring compounds for the functional assay based on the energy scores calculated by molecular docking. The energy scores of these compounds varied between $-16$ and $-20$ kcal/mole and their molecular weights fell in a small range (Table 1).

Subsequently, DOCK5.1 (UCSF) was used to position $\beta$-ionone and other compounds. The orientations of retinal from the x-ray structure and $\beta$-ionone as posed by DOCK5.1 are depicted in Figures 3B and 3C. The solvent-accessible surface of the retinal-binding pocket is depicted as a mesh, where green represents a preponderance of carbon atoms; blue, nitrogen; red, oxygen; and orange, sulfur. Based on the orientation posed by molecular docking, $\beta$-ionone interacts with the same area of the pocket where the cyclohexyl head group of the retinal binds.

**Compound NSC45012 Inhibits Opsin Regeneration and Rescues Misfolded P23H Opsin**

To validate the in silico approach, we first tested the five top-scoring compounds for their effectiveness as inhibitors of opsin regeneration. Of these five, only one compound, NSC45012 [1-(3,5-dimethyl-1H-pyrazol-4-yl) ethanone], (Fig. 4A) showed a significant effect on inhibition of pigment formation with retinal (Fig. 5A). This compound consists of a five-carbon pyrazol ring with methyl groups at positions 3 and 5 and a ketone group at the terminus. The orientation of NSC45012 determined by DOCK5.1 showed that it also occupies the same hydrophobic space of the retinal binding site as the six-membered ring of retinal (Fig. 4B). On superimposing the orientations of retinal and NSC45012, we found the methyl groups of the small molecule to be oriented along the same

![Figure 3](https://tvst.arvojournals.org/)  
**FIGURE 3.** Molecular docking strategy for pharmacologic chaperones of rhodopsin. (A) The crystal structure of rhodopsin is shown with yellow spheres representing bound retinal. The site selected for molecular docking is encompassed by a scoring grid shown as a white box. $\alpha$-Helices, red; $\beta$-sheets, yellow; loop regions, green. (B, C) Retinylidene and $\beta$-ionone are shown in the retinal-binding pocket in the orientation posed by molecular docking. Cyan, carbon; gray, hydrogen; blue, nitrogen; red, oxygen. (B) Only Lys296 of the retinal binding pocket is shown in sticks (green), whereas the remainder of the binding pocket is depicted as mesh.

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space as the ring methyl groups of retinal, but their positions were not identical (Fig. 4C). These data may suggest the importance of these methyl groups in the formation of weak hydrophobic linkages. The molecular graphics program revealed the amino acid groups of the retinal binding site closest to the small molecule (Figs. 4D, 4E). These groups are schematically depicted in Figure 4F, and the probable weak hydrophobic linkages in which they may participate with NSC45012 are also shown.

In the in vitro competition assay, NSC45012 inhibited rhodopsin formation in a dose-dependent manner. Preincubation of opsin with this compound at 2 or 5 mM increased the $t_{1/2}$ of regeneration with retinal from 5 minutes to 8 and 12 minutes, respectively (Fig. 5A). This compound is a weaker inhibitor than $\beta$-ionone, because higher concentrations were required to yield equivalent levels of inhibition. No pigment was detected when NSC45012 was combined with opsin in vitro. The pure compound did not show any absorption in the visible spectrum (Fig. 5B).

We next evaluated its ability to rescue P23H opsin. A 1.3-fold increase in the yield of folded P23H rhodopsin was achieved in the presence of NSC45012, as measured by UV-
visible spectrometry (Fig. 5C). The increase in yield is lower than that achieved with β-ionone.

**DISCUSSION**

In this study, we showed that in silico computational methods can be coupled to biochemical assays to develop an efficient screening method capable of identifying interacting molecules for rhodopsin even when the interaction is weak. We also demonstrated a strong correlation between the efficiency of binding of a molecule to the retinal binding site, as measured by inhibition of pigment formation and its potency as a pharmacologic chaperone.

In agreement with previous reports, our in vitro experiments with β-ionone showed a robust inhibition of pigment formation. β-Ionone has the same six-membered ring configuration of retinal, but a considerably shorter side chain that does not allow formation of the covalent bond. It has been shown that β-ionone inhibits access of retinal into the binding site by anchoring through its cyclohexyl head, where weak hydrophobic interactions play an important role and the presence of the ring methyl groups is critical. In keeping with the importance of the length of the polyene chain, cis-1,3-dimethylcyclohexane, which has the six-carbon head group, two methyl groups but no polyene chain, showed much weaker inhibition than β-ionone. The compound identified in this study, NSC45012, has a five-membered ring, a ketone group, and no polyene chain. Of note, it contains three methyl groups that occupy positions very similar to those of ring methyl groups in retinal. The presence of a ketone group is another similarity that NSC45012 shares with β-ionone. Both β-ionone and NSC45012 occupy the same hydrophobic region of the retinal binding site. The major differences in the ring structure and the lack of a polyene chain may chemically explain its lesser inhibitory effect.

We demonstrated that these inhibitors act as pharmacologic chaperones for P23H opsin. β-Ionone showed a 2.5-fold rescue of P23H pigment. The formation of pigment on addition of retinal to β-ionone- and NSC45012-treated cells also demonstrated that the retinal could competetively displace these competitors from the retinal binding site. The noncovalent, weak hydrophobic association between these compounds and the chromophore-binding site provides an important advantage. They can be readily replaced by the much stronger interaction between 11-cis retinal and opsin once the folded protein arrives in the outer segment. The easy dissociation of the rescuing agent from the target molecule is a desirable trait for a pharmacologic chaperone.

Our results also demonstrate a strong relationship between the potency of the inhibitor and its ability to function as a pharmacologic chaperone. The maximum level of rescue of P23H opsin was achieved with the natural ligand 11-cis retinal, probably because of the formation of stable Schiff base linkage. Such a correlation between binding and pharmacologic efficacy has been seen with variants of the human gonadotropin-releasing hormone receptor (GnRHR), another GPCR, and a human ether-a-go-go-related gene (HERG) K+ channel mutant. The good correlation between binding and efficacy observed in our study may be due to the use of Lipinski rules of drug likeness.

The approach used in this study made use of a combination of high-throughput computational screening, biochemical characterization, and cell-based assays. A similar approach has been used in which in silico screening was successfully coupled with flow cytometry to identify small molecule antagonists of the formylpeptide receptor, also a GPCR. Despite many challenges and shortcomings, docking and scoring technology has been successfully used for identifying novel bioactive molecules, and we showed that it can be very effectively used to identify even weakly interacting ligands for rhodopsin.

The retinoids, being related structurally to retinal, could be easily screened with the in vitro competition assay, but selection of nonretinoids becomes much easier with the coupling of the in silico method. The small molecules identified through this combination approach could be further modified and re-tested by the same biochemical and cell-based methods as described herein. Although only one mutant was studied in this investigation, it is reasonable to expect that the pharmacologic chaperones identified through this approach would be beneficial for other misfolding rhodopsin mutants as well.

The broad significance of these findings is that this combinatorial high-throughput screening method can be applied to RP and other diseases of protein folding and may yield potential therapeutic molecules.

**References**

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