Pharmacological clearance of misfolded rhodopsin for the treatment of RHO-associated retinitis pigmentosa

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Abstract
Rhodopsin mutation and misfolding is a common cause of autosomal dominant retinitis pigmentosa (RP). Using a luciferase reporter assay, we undertook a small-molecule high-throughput screening (HTS) of 68,979 compounds and identified nine compounds that selectively reduced the misfolded P23H rhodopsin without an effect on the wild type (WT) rhodopsin protein. Further, we found five of these compounds, including methotrexate (MTX), promoted P23H rhodopsin degradation that also cleared out other misfolded rhodopsin mutant proteins. We showed MTX increased P23H rhodopsin degradation via the lysosomal but not the proteasomal pathway. Importantly, one intravitreal injection (IVI) of 25 pmol MTX increased electroretinogram (ERG) response and rhodopsin level in the retinae of RhoP23H/+ knock-in mice at 1 month of age. Additionally, four weekly IVIs increased the photoreceptor cell number in the retinae of RhoP23H/+ mice compared to vehicle control. Our study indicates a therapeutic potential of repurposing MTX for the treatment of rhodopsin-associated RP.

KEYWORDS
high-throughput screening, methotrexate, misfolded protein degradation

Abbreviations: Ad, autosomal dominant; AHA, azidohomoalanine; BaF1A1, Bafilomycin A1; BTN, biotin; bw, body weight; DDM, n-Dodecyl-β-D-maltopyranoside; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; ER, endoplasmic reticulum; ERG, electroretinogram; HIF1-α, heat shock-induced factor 1α; FDA, US food and drug administration; HRP, horseradish peroxidase; HTS, high-throughput screening; IHC, immunohistochemistry; IVI, intravitreal injection; LC3, microtubule-associated proteins light chain 3; LOPAC, library of pharmacologically active compounds; MIPE, mechanism interrogation plat E; MTX, methotrexate; ONH, optic nerve head; ONL, outer nuclear layer; OS, outer segments; PBS, phosphate-buffered saline; PND, postnatal day; Rluc, Renilla luciferase; RP, retinitis pigmentosa; SA, streptavidin; SQSTM1, sequestosome 1; UC, University of Cincinnati; WT, wild type.

Xujie Liu and Bing Feng are the authors contributed equally to this work.
1 | INTRODUCTION

Protein misfolding and unfolded protein response are found to contribute to inherited retinal diseases such as retinitis pigmentosa (RP), a progressive retinal degeneration affecting more than one million people worldwide. The disease progression of RP varies widely but can last for decades. Gradual loss of rod photoreceptor cells causes night blindness followed by reduced visual field and finally tunnel vision. The central vision of many RP patients can last for many years until the secondary loss of cone photoreceptors when legal blindness occurs. More than 160 mutations in the RHO gene have been associated with RP (www.hgmd.cf.ac.uk), and about 1/3 of these mutations are believed to cause rhodopsin misfolding leading to a dominant-negative effect that is toxic to the rod photoreceptor cells. The RHO P23H mutation alone accounts for about 10%-12% of all autosomal dominant (ad) RP cases in North America, and thus, this mutation has been most commonly studied as a model of adRP. Like other protein misfolding diseases, no effective treatment is currently available for RP.

The dim-light receptor rhodopsin is the most abundant protein residing in the outer segments (OS) of rod photoreceptor cells and supports high visual sensitivity at night. Rhodopsin homeostasis is essential to maintain the rod OS morphology and rod photoreceptor function. Due to its high abundance and a 10% daily renewal rate of rod OS, rhodopsin biosynthesis is maintained at an extremely high level to keep rod OS length being constant. Thus, even one allele of RHO gene mutation can substantially disturb rhodopsin protein homeostasis, leading to rod cell death in RP. The P23H mutation affects the structural stability of the antiparallel β-plug scaffold sitting on top of the retinal-binding site of rhodopsin, and this β-plug scaffold is essential for excluding the hydrophobic ligand-binding pocket from aqueous environment. The mutant rhodopsin protein accumulates in the endoplasmic reticulum (ER) in cultured cells. The ER-associated protein degradation pathway is activated in the Rhop23H/+ knock-in mouse retina, and more than 90% of the mutant rhodopsin protein undergoes degradation, supporting the notion that the protein quality control system is working hard to maintain rhodopsin homeostasis. Nonetheless, this robust proteolytic system in the rods of the Rhop23H/+ mouse retina is in the long-term overwhelmed by the constant and high load of rhodopsin degradation.

To prevent the misfolded rhodopsin-caused rod death in early- or mid-stage adRP, experimental efforts have been focused on supporting rhodopsin folding or boosting the ER-associated protein degradation system. For example, pharmacological or chemical chaperones were reported to improve rhodopsin folding and its cellular transport, including the vitamin A derivatives and analogues, 4-phenylbutyrate and curcumin. High-dose vitamin A supplementation has shown some level of visual protection among RP patients. However, due to the lack of genetic information of these patients, it is not clear whether the efficacy of vitamin A is due to an increased retinal supply of 11-cis-retinal as a pharmacological chaperone of rhodopsin. We recently identified a non-retinoid ligand that stabilizes rhodopsin folding, improves the glycosylation profile of the P23H rhodopsin, and shows retinal protection in a light damage mouse model of retinal degeneration. Importantly, the chaperone activity of these non-retinoid ligands, unlike 11-cis-retinal, are independent of light, and thus, they will serve as chaperones as long as they are delivered to the retina. Alternatively, rhodopsin homeostasis has also been shown to be rescued by modulating the endogenous protein quality control system using heat shock protein 90 inhibitors, a heat shock response inducer, and an activator of eukaryotic initiation factor 2α, suggesting the endogenous molecular support of protein folding is not sufficient in RHO-associated RP.

Instead of support folding, reducing the misfolded rhodopsin has been shown as an effective strategy to rescue rod photoreceptors. Long-term retinal protection has been shown in P23H transgenic rats that were treated by gene delivery of a small ribozyme that specifically cut the mutant allele of rhodopsin mRNA. Enhancing misfolded rhodopsin degradation by transgenic overexpression of a regulatory subunit of proteasome has also shown retinal protection in the rhodopsin P23H knock-in mice. These studies suggest clearing the misfolded rhodopsin is sufficient to preserve rod photoreceptors in RHO-associate adRP. However, no effective pharmacological tools are available to clear the misfolded rhodopsin and show retinal protection in vivo. In this study, we targeted rhodopsin homeostasis in the rod cells expressing a mutant rhodopsin by small molecules as a preventive treatment of RHO-associated adRP because this is the first cellular event being disrupted that causes rod stress and death. Here, we have identified an approved drug methotrexate (MTX) by high-throughput screening (HTS) with a cell-based assay that selectively accelerated P23H rhodopsin degradation via lysosomal activity. Importantly, MTX improved rhodopsin homeostasis and increased visual function by a single intravitreal injection (IVI) in the Rhop23H/+ knock-in mice. Further, multiple weekly IVIs of MTX led to higher photoreceptor cell numbers on the superior retinae of the Rhop23H/+ knock-in mice compared to vehicle control. The activity of MTX in inducing misfolded rhodopsin clearance implicates its potential in treating inherited retinal degenerations caused by protein misfolding.

2 | MATERIALS AND METHODS

2.1 | Stable cell lines

Two Hek293 stable cell lines, Hek293 (RHOWT-Rluc) and Hek293 (RHO23H-Rluc) cells were generated which stably
express the wild type (WT) and P23H mutant mouse rhodopsin each fused with a Renilla luciferase (Rluc) for the luciferase reporter assay as previously described. Briefly, the Hek 293 cells (ATCC, Manassas, VA, USA) were transfected with the pcDNA3.1 Zeo containing the cDNA of mouse WT or P23H rhodopsin fused with a Rluc 8 (a gift from Dr Navine Lambat at Augusta University, Augusta, GA, USA) and transfected cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM, Genesee Scientific, El Cajon, CA, USA) with 10% of fetal bovine serum (FBS, Gibco Laboratories, Gaithersburg, MD, USA) for 48 hours before addition of 400 μg/mL of Zeocin (InvivoGen, San Diego, CA, USA) for positive clone selection. Colonies of cells survived from 1 week of Zeocin selection were collected, and expression of WT and P23H rhodopsin were confirmed by a luciferase assay and immunoblotting of rhodopsin with positive bands of monomers at about 70 kD (Supplementary Figure S1). The difference of molecular masses between WT and P23H rhodopsin proteins was due to differences in glycosylation, which was also seen in the NIH3T3 cells expressing the WT and P23H rhodopsin proteins.

Two NIH3T3 stable cell lines, NIH3T3 (RHOWT/GFP) and NIH3T3 (RHOP23H/GFP) were shared by Dr Krzysztof Palczewski’s lab who generated them by incorporating the pMiLRO 23 and pMiLRO DNA constructs to the NIH3T3 Palczewski’s lab who generated them by incorporating the pMiLRO 23 and pMiLRO DNA constructs to the NIH3T3 cells, respectively, via viral transduction. GFP was co-expressed with rhodopsin for positive-clone selection. The expression of the WT and P23H rhodopsin protein was confirmed by immunoblots and immunostaining.

Seven U2OS stable cell lines were generated which separately express the WT and six mutant mouse rhodopsin (T4R, P23H, P53R, C110Y, D190N, and P267L) fused with Venus fluorescence protein. Briefly, the U2OS cells (ATCC, Manassas, VA, USA) were transfected with the pcDNA3.1 Zeo containing the cDNA of mouse WT or T4R, P23H, P53R, C110Y, D190N, and P267L rhodopsin fused with Venus and transfected cells were incubated in DMEM with 10% of FBS for 48 hours before addition of 400 μg/mL of Zeocin (InvivoGen, San Diego, CA, USA) for positive clone selection. Colonies of cells survived from 1 week of Zeocin selection were collected, and expression of WT and six mutant rhodopsins were confirmed by fluorescence of Venus and immunoblotting of rhodopsin.

2.3 | Chemicals and reagents

ViviRen was purchased from Promega (Madison, WI, USA), dissolved in dimethyl sulfoxide (DMSO) as 60 mM stock aliquots and stored at −80°C in amber tubes. The UC 10 k Diversity Set was provided by the University of Cincinnati Drug Discovery Center (UCDDC, Cincinnati, OH, USA) at 10 mM per compound in 384-well format, the Spectrum Collection (MicroSource, Gaylordsville, CT, USA) and the Life Chemicals 50K Diversity Set (Life Chemicals USA, Woodbridge, CT, USA) were provided by Dr Krzysztof Palczewski at 10 mM per compound in 384-well format, and the US Food and Drug Administration (FDA) approved drugs, Library of Pharmacologically Active Compounds (LOPAC, Millipore Sigma, St. Louis, MO, USA), and Mechanism Interrogation Plat E (MIPE) collections were provided by National Center for Advancing Translational Sciences (NCATS, Bethesda, MD, USA) in 7 or 11 dose series for each compound in 1536-well format. All the compounds in these compound libraries were dissolved in DMSO and stored at −80°C, and sealed with adhesive foil films. Hit compounds were cherry-picked from the compound stock in powder or ordered from chemical vendors for triplicate, dose-response tests, as well as confirmation and counter screens. CL-001 (PubChem CID:11715767), CL-006 (CID:11338033), CL-007 (CID:5330790), CL-008 (CID:16747683), Bafilomycin A1 (BafA1), and MG-132 were purchased from Selleckchem (Houston, TX, USA); CL-002 (CID:6224422), CL-003 (CID:4438424), and CL-004 (CID:6624030) were ordered from Life Chemicals, CL-005 (CID:10091681) was provided by UCDDC, and CL-009/MTX (CID: 126941) were purchased from Cayman Chemical (Ann Arbor, MI, USA). DMSO and L-Met were from MilliporeSigma (St. Louis, MO, USA). L-Met-free DMEM was from Gibco (21013-024, Gaithersburg, MD, USA). The anti-rhodopsin antibodies 1D4 and B630 were shared by Dr Krzysztof Palczewski’s lab. Anti-microtubule-associated proteins light chain 3 (LC3) antibody was purchased from Cell Signaling Technology (4108S, Danvers, MA, USA). Anti-sequestosome 1 (SQSTM1/p62) antibody was purchased from Novus Biologicals (NB1-42821, Centennial, CO, USA). Cy3-conjugated goat anti-mouse IgG (A10521), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (32230), HRP-conjugated goat anti-rabbit IgG (32260), HRP-Streptavidin (SA) (434323), L-azidohomoalanine (AHA, C10102), bortezomib (BTN)-s-DIBO (C20023), Dynabeads Protein G (10004D), BCA protein assay (23225), paraformaldehyde (28908), Hoechst 33342 (H3570), TRIZol (15596026), nuclease free water (BP2484-100), high-capacity RNA-to-cDNA kit (438740), PowerUp SYBR Green Master Mix (A25741) and SuperSignal West Pico PLUS Chemiluminescent Substrate (34580) were obtained from Thermo Fisher (Waltham, MA, USA).

2.2 | Cell culture and media

Cells were cultured in the complete medium containing DMEM with 10% of FBS and 5 μg/mL of plasmocin (InvivoGen, San Diego, CA, USA) at 37°C with 5% CO₂ and > 95% humidity, and subcultured as instructed in the ATCC Animal Cell Culture Guide (www.atcc.org).
2.4 HTS and counter screening procedures

The HTS was performed at three facilities, including University of Cincinnati (UC), Case Western Reserve University, and NCATS, testing a total of six compound collections. These compound collections include: (1) the UC 10K Diversity Set (10,011 compounds), (2) the Life Chemicals 50K Collection (50,560), (3) the Spectrum Collection (2400 compounds), (4) the LOPAC collection (1280 compounds), (5) the FDA collection (2816 compounds), and (6) the NCATS MIPE collection (1912 compounds). The procedure of the HTS assay and confirmation assays are slightly different (Supplementary Table S1) to adapt to equipment at different facilities, nonetheless the quality control parameters (signal to noise ratio and Z’ factors) demonstrate these assays were robust (Table 1). For the compound collections (1), (2), and (3), we first tested each compound using the P23H rhodopsin luciferase reporter assay at a single dose for once (9.93 to 16.13 μM) in 384-well format, and we identified 2072 compounds with activity scores lower than the cutoff at mean-2SD (Supplementary Data 1). Activity scores were normalized using DMSO-treated cells as 0% control and the 1 mM Evans Blue-treated cells as −100% control. From these hit compounds, we excluded those reported with luciferase inhibitor activities and cherry-picked the rest for hit confirmation. Each hit was tested at 10 μM in the Hek293(RHO<sup>P23H</sup>)-Rluc cells again by the luciferase reporter assay in triplicates, and the confirmed hits with activity scores lower than −50% were then counter screened for their effects on Rluc activity by the recombinant Rluc activity assay, and on WT rhodopsin level by the luciferase reporter assay in the Hek293(RHO<sup>WT</sup>)-Rluc cells at 10 μM in triplicates, respectively. We identified 52 compounds that do not significantly affect the Rluc activity or the WT rhodopsin-Rluc reporter activity (activity scores higher than −50%) (Supplementary Data 1). Next, we tested the dose responses of these confirmed hits in both the Hek293(RHO<sup>P23H</sup>)-Rluc and the Hek293(RHO<sup>WT</sup>)-Rluc cells and identified compounds that selectively favored the clearance of P23H than WT rhodopsin in a dose-dependent manner (Supplementary Table S1).

For the compound collections (4)-(6), we tested each compound directly at 7 or 11 doses using the luciferase reporter assay in the Hek293(RHO<sup>P23H</sup>)-Rluc cells in 1536-well format, and identified 128 compounds with efficacy smaller than −50% and EC<sub>50</sub> < 20 μM. We then cherry-picked these 128 compounds and tested them by the luciferase reporter assay in both Hek293(RHO<sup>P23H</sup>)-Rluc and Hek293(RHO<sup>WT</sup>)-Rluc cells in 7 or 11 doses in triplicates and selected 34 compounds that showed higher efficacy toward the clearance of P23H than WT rhodopsin (Supplementary Data 1).

**Table 1** The quality control parameters of the HTS of each compound collection using the luciferase reporter assay

| Number | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---|---|---|---|---|---|
| Compound library | 10K UC collection | Spectrum collection | Life chemicals 50K collection | FDA collection | LOPAC | MIPE |
| Location | University of Cincinnati | Case Western Reserve University | Case Western Reserve University | NCATS | NCATS | NCATS |
| Number of compounds | 10,011 | 2400 | 50,560 | 2816 | 1280 | 1912 |
| Tested concentration (μM) | 9.93 | 12.99 | 16.13 | 7 doses | 7 doses | 11 doses |
| Z’ range | 0.53-0.71 | 0.54-0.84 | 0.44-0.87 | 0.37-0.61 | 0.52-0.71 | 0.46-0.66 |
| Z’ mean ± SD | 0.62 ± 0.05 | 0.71 ± 0.10 | 0.67 ± 0.10 | 0.50 ± 0.07 | 0.63 ± 0.07 | 0.59 ± 0.05 |
| S/B range | 13.07-51.33 | 8.27-51.33 | 70.1-280.5 | 137.24-158.20 | 94.63-184.52 | 145.97-192.00 |
| S/B mean ± SD | 19.81 ± 23.36 | 19.81 ± 19.36 | 140.9 ± 26.3 | 148.21 ± 7.94 | 124.77 ± 28.97 | 159.67 ± 13.66 |
| 0% | DMSO 0.1% | DMSO 0.1% | DMSO 0.1% | DMSO 0.1% | DMSO 0.1% |
| −100% | Evans Blue 200 μM | Evans Blue 1 mM | Evans Blue 1 mM | Evans Blue 1 mM |
| Cutoff (Mean-2SD) (%) | −50 | −32.25 | −49.76 | EC<sub>50</sub> < 20 μM |
| Number of hits | 82 | 89 | 1901 | 34 |
| Hit rate (%) | 0.82 | 3.71 | 3.76 | 0.57 |

*Note:: Z’=1-3 × (SD<sub>0%</sub> + SD<sub>−100%</sub>)/(Mean<sub>0%−Mean−100%</sub>).*

*Abbreviations: S/B, signal-to-background ratio. S/B = Mean<sub>0%</sub>/Mean<sub>−100%</sub>.*
2.5 | Rluc reporter assay

The Rluc reporter assay has been described before. HTS was undertaken in 384-well format for the UC 10K Diversity Set, Spectrum Collection, and the Life Chemicals 50K diversity set, and in 1536-well format for the FDA, LOPAC, and MIPE collections. Using the 384-well format as an example, Hek(RHOP23H-Rluc) cells were seeded in 384-well white-wall clear-bottom plates (assay plates) at 3 \times 10^5 cells/mL for 40 μL/well by an 8-channel MultiFlo liquid dispenser (BioTek, Winooski, VT, USA). The assay plates were centrifuged at 300 \times g for 30 seconds and cultured at 37°C with 5% CO_2 overnight. On the next day, compounds were transferred by a 50 nL 384-pin tool from 384-well compound plates to 384-well assay plates that contained cultured cells, operated by a JANUS MDT automatic workstation (PerkinElmer, Waltham, MA, USA). Compound plates contained 50 μL/well of compound solutions dissolved in DMSO in columns 3-22. Controls were loaded in a control plate in columns 1, 2, 23, and 24, containing 50 μL of DMEM, DMSO, DMSO, and Evans Blue (606 mM in DMSO), respectively. Controls were transferred using the 50 nL pin tool from the compound plate to the assay plate. Before reuse, the pin tool was washed thoroughly by sequentially dipping into wash wells containing 100 mL of DMSO, flushing water, and 100 mL of ethanol and air-dried for 30 seconds. Treated assay plates were centrifuged at 300 × g for 30 seconds and incubated at 37°C with 5% CO_2 for 24 hours. On the third day, 5 μL/well of 2% n-Dodecyl-β-D-maltopyranoside (DDM) was added to the assay plates followed by 5 seconds of shake. The assay plates were incubated at room temperature for 5 minutes and added with 5 μL/well of 50 μM ViviREN solution diluted in phosphate-buffered saline (PBS), followed by 5 seconds of shake. The assay plates were incubated in dim light at room temperature for 1 hours. The luminescence of each well was read by an Enspire plate reader (PerkinElmer) with 0.1 seconds of integration time. For the 1536-well format of the HTS assay, the procedures are same as the 384-well assay, except for the following differences: cells were seeded at 3000 cells/well in 5 μL/well of a white-wall clear-bottom 1536-well plate; compounds were transferred using a 23 nL.1536-pin tool; and 0.5 μL/well of DDM and 1 μL/well of 30 μM ViviREN solution were sequentially added to the assay plates. For the counter screening assay, the assay was repeated as the HTS assay using the Hek293 (RhōWT-Rluc) cells. Activity score (%) = (RLU_{compound}−RLU_{DMSO})/(RLU_{DMSO}−RLU_{Evans Blue}) \times 100. RLU, relative luminescence unit.

2.6 | Recombinant Rluc activity assay

Recombinant Rluc (RayBiotech, Peachtree Corners, GA, USA) was dissolved in PBS and diluted to 0.3 μg/mL. Each compound was diluted to 10× final concentration in PBS as the compound working solution. In 384-well plates, 16 μL/well of diluted Rluc was mixed with 4 μL/well of compound working solution. Using a SpectraMax XL microplate reader (Molecular Devices, San Jose, CA, USA), luminescence in each well was read 2.5 seconds after an injection of 20 μL/well of 5 μM of coelenterazine h substrate (Nanolight Technologies, Pinetop, AZ, USA). Each compound was tested at a final concentration of 10 μM and repeated three times. Luciferase activity was normalized by the DMSO and 1 mM Evans Blue as 0% and −100% controls, respectively.

2.7 | Dot blot

NIH3T3 (RhōP23H/GFP) or NIH3T3 (RhōWT/GFP) cells were seeded at 2.5 × 10^4 cells/well in 100 μL/well complete medium in a 96-well plate and cultured at 37°C with 5% CO_2 for 4 hours. Cells then were treated with 100 μL/well complete medium containing 2× the final concentration of tested compounds. After 24 hours of further incubation at 37°C with 5% CO_2, the medium was aspirated, and the cells were washed once with PBS. The cell lysis buffer containing radioimmuno-precipitation assay (RIPA) buffer and complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) were added to the cells at 200 μL/well followed by sonication for 6 seconds. Because the cellular protein level of RhōWT was higher than RhōP23H, 5% of one well of NIH3T3 (RhōWT/GFP) cells samples and 90% of one well of NIH3T3 (RhōP23H/GFP) cells samples were loaded to a nitrocellulose membrane and air-dried. Rhodopsin protein was immunostained with 0.1 μg/mL of HRP-conjugated 1D4 anti-rhodopsin antibody. Densitometry of the bands was measured by the ImageJ software and normalized to DMSO-treating control.

2.8 | Western blot

Cells were seeded in 6-well plates at a density of 5 \times 10^5 cells/well and cultured at 37°C with 5% CO_2 for 17 hours. The medium was replaced with a fresh medium containing a corresponding concentration of compounds. After treatment for 24 hours, cells were collected and lysed in 150 μL PBS containing 0.1% of SDS and complete protease inhibitor cocktail with 12 seconds of sonication on ice. Retina samples were lysed in 300 μL per retina of PBS containing 0.1% of SDS and complete protease inhibitor cocktail under 24 seconds of sonication on ice. Protein concentrations were determined by measurements of OD_{280 nm} using a NanoDrop spectrometer. To immunoblot rhodopsin, 100 μg of total protein for NIH3T3 (RhōP23H/GFP) cells, or 20 μg of total protein for NIH3T3 (RhōWT/GFP) cells was loaded per well onto an SDS-PAGE gel. To detect other proteins, 50 μg of total protein was loaded per well. After separation by electrophoresis
in 10% or 16% SDS-PAGE gels, proteins were transferred to a nitrocellulose membrane using a wet membrane electrophoretic transfer cassette followed by blocking with 5% of bovine serum albumin in PBS containing 0.05% of Tween 20 for 1 hour. The membranes were incubated with primary antibodies at 4°C overnight following with appropriate secondary antibodies for 1 hour at room temperature. Blots were visualized using SuperSignal West Pico PLUS chemiluminescent substrate, and scanned by a BioRad gel imager.

2.9 High content imaging

To assess the effect of active compounds on the clearance of P23H rhodopsin in mammalian cells, an image-based assay was performed using NIH3T3 (RHO\textsuperscript{WT}/GFP) and NIH3T3 (RHO\textsuperscript{P23H}/GFP) cells, as described previously. Briefly, cells were seeded at 5000 cells/well in 384-well plates and incubated at 37°C in 5% CO\textsubscript{2} for 4 hours until cells were attached to the bottom of the plate. Attached cells were treated with compounds for 24 hours. The assay medium was aspirated, and cells were fixed with 4% of paraformaldehyde at 20 μL/well for 20 minutes at room temperature. Cell membranes were permeabilized with PBS containing 0.1% of Triton X-100 (PBST), and then, incubated with 50 μg/mL of 1D4 anti-rhodopsin antibody at 15 μL/well for 1 hour at room temperature. After three washes with 50 μL/well of PBST, cells were incubated with 15 μL/well Cy3-conjugated goat anti-mouse IgG antibody for 1 hour at room temperature. After three washes with 50 μL/well of PBST, cells were incubated with 15 μL/well Cy3-conjugated goat anti-mouse IgG antibody for 1 hour at room temperature. After three washes with 50 μL/well of PBST, cells were incubated with 15 μL/well Cy3-conjugated goat anti-mouse IgG antibody for 1 hour at room temperature.

2.11 Pulse-chase assay

To quantify rhodopsin degradation, a non-radioactive pulse-chase assay was used with a “click” reaction. Briefly, NIH3T3 (RHO\textsuperscript{P23H}/GFP) or NIH3T3 (RHO\textsuperscript{WT}/GFP) cells were cultured in a 24-well plate at 3 x 10\textsuperscript{5} cells/well in the complete medium with 10% of FBS. The medium was aspirated after overnight culture, and cells were gently washed once with 1 mL/well of PBS. Cells were incubated in the L-Met-free DMEM for 1 hour to exhaust the intracellular Met. Then, the cells were pulse with L-AHA at 50 μM for 4 hours. Meanwhile, the cells treated with 50 μM of L-Met were used as negative control. After labeling, cells were replaced with the complete DMEM medium with 2 mM of L-Met in the presence or absence of active compounds for a varying time of chase. Then, cells were lysed with PBS containing 0.1% of SDS, 1% of DDM, and the complete protease inhibitor cocktail. The total protein concentrations were measured by a BCA assay. Cell lysate containing 200 μg total protein was mixed with 5 μM of BTN-sDIBO to make a “click” reaction for 1 hour at 37°C. Samples were incubated with Dynabeads Protein G binding with 1D4 anti-rhodopsin antibody for 15 minutes at room temperature followed by three washes with PBS containing 0.02% of Tween 20. Proteins were eluted with 50 mM of Glycine at pH 2.8 for 10 minutes at room temperature, and then, loaded onto nitrocellulose membranes. After air-dried, the membranes were
blocked with 5% of milk and immunoblotted with 0.25 μg/mL of HRP-conjugated SA.

2.12 | Proteasome activity assay

NIH3T3(RhoP23H/GFP), NIH3T3(RhoWT/GFP), and NIH3T3 cells were seeded in a white-wall clear-bottom 384-well plate at 2500 cells/well in 20 μL of complete medium. After 3 hours of incubation, 5 μL/well of complete medium containing MTX was added to cells to treat cells with MTX at final concentrations from 0.0195 to 10 μM for 24 hours. The cells treated with a complete medium containing 0.1% of DMSO were used as the 100% control, and those treated with 5 μM of MG-132 for 8 hours were used as the 0% control. For endpoint proteasome activity measurement, each well was added with 25 μL of Proteasome-Glo Reagent containing the Suc-LLVY-Glo substrate (Promega, G8660, Madison, WI, USA). The 384-well plate was shaken for 2 minutes to mix the solutions followed by a 24-minutes incubation at room temperature. Luminescence of each well was detected by a SpectraMax I3X microplate reader (Molecular Devices). The chymotrypsin-like proteasome activity was normalized by the 100% and 0% controls, respectively.

2.13 | Animals

The C57BL/6J (Rho+/+) mice and RhoP23H/P23H mice were purchased from Jackson Laboratory (Stock No 017628) generated by Dr Krzysztopalczewski’s lab. The RhoP23H/P23H mice were crossed with WT C57BL/6J mice to produce P23H heterozygotes mice. Genotyping for all strains was conducted as guided using forward and reverse primers: (i) GGT AGC ACT GTT GGG CAT CT; and (ii) GAC CCC ACA GAG ACA AGC TC, respectively. The PCR products at 573 and 399 bp indicated the P23H knock-in mutant and WT allele of the Rho gene, respectively. Mice were bred and housed under standard 12-h light/12-h dark conditions in the University of Pittsburgh animal facility. All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) following the guide for the Animal Welfare Act and Regulations.

2.14 | Intravitreal injection (IVI)

To determine the effect of compounds on retina in vivo, we administered compounds directly into the vitreous space. Briefly, mice were treated with 1% tropicamide eye drops (Akorn, Lake Forest, IL, USA) to dilate the pupils, and then, they were anesthetized with an intraperitoneal injection of ketamine (Henry Schein, Dublin, OH, USA) at 80 mg/kg body weight (bw) and xylazine (Bimeda, Le Sueur, MN, USA) at 7 mg/kg bw. One drop of 0.5% tetracaine hydrochloride (TCI, Tokyo, Japan) was applied to mouse eyes as topical anesthetics before injection. Eyes were kept lubricated during the injections by a 0.3% hypromellose eye gel (Alcon, Fort Worth, TX, USA). A heating pad was used for maintaining body temperature. Mice were positioned to expose the sclera of the eyes. A 30-gauge needle (Medline, Northfield, IL, USA) was used to puncture a hole through sclera behind limbus at a 45° angle. A 33-gauge blunt-end needle (Hamilton, Reno, NV USA) was used to be inserted into the hole and a total of 0.5 μL of sterile PBS or MTX in PBS was slowly injected into the posterior chamber and the needle was kept in place for about 30 seconds before a slow removal of the needle. A small amount of Triantibiotic ointment (Medline, Northfield, IL, USA) was used on the injection site to prevent infection. A single IVI with 25 or 100 pmol of MTX was administered to one eye of each RhoP23H/+ mouse at postnatal day (PND) 15, and PBS was injected to the other eye as vehicle control. We also performed four weekly IVIs of MTX or PBS to the second group of RhoP23H/+ mouse eyes at PND 15, 22, 29 and 36.

2.15 | Electroretinogram (ERG)

ERG was performed using the Celeris system (Diagnosys, Lowell, MA, USA), as described previously. Before each test, mice were kept in the dark overnight. Pupils were dilated with 1% tropicamide (Akorn, Lake Forest, IL, USA). Mice were anesthetized with an intraperitoneal injection of ketamine at 80 mg/kg bw and xylazine at 7 mg/kg bw. Eyes were lubricated by a 0.3% hypromellose eye gel (Alcon, Fort Worth, TX, USA). A heating pad was used to maintain body temperature at 37°C. Scotopic ERG responses of dark-adapted eyes to eight flashes from 0.01 cd/s/m² to 30 cd/s/m² were recorded and averaged from three sweeps per flash intensity with intersweep intervals of 10 to 30 seconds. After exposed to 10 cd/m² illumination for 5 minutes, the photopic ERG responses were recorded from the light-adapted eyes in respond to flashes from 0.01 cd/s/m² to 30 cd/s/m² in addition to a 10 cd/m² background light. P values were calculated by a two-way ANOVA to determine the statistical significance between the response amplitudes in the MTX-treated and vehicle (PBS) group. Factor 1, treatment condition; and factor 2, flash intensity.

2.16 | Tissue collection and immunohistochemistry (IHC)

Mice were euthanized and the superior side of each eye was labeled by a burn mark generated by a Cautery pen. Eyes
were enucleated by a pair of curved-tip forceps and fixed in the freshly prepared 4% paraformaldehyde for 2 hours. Fixed eyes were dehydrated sequentially in 5%, 10%, 20%, and 40% sucrose solutions in PBS, each for 30 minutes at room temperature. Finally, eyes were incubated in a mixture of 40% sucrose in PBS and embedding medium for frozen tissue specimens to ensure optimal cutting temperature (OCT compound, FisherScientific, Houston, TX, USA) at 1:1 volume ratio for overnight at 4°C before they are embedded in the same mixed solution in an orientation-specific manner and frozen in liquid nitrogen-bathed isobutene. Twelve-micron retinal cross-sections were made by a microtome at −16°C and those containing the optic nerve head (ONH) were applied onto a SuperFrost glass slide (FisherScientific). These slides were then used for IHC. After rehydration and permeabilization in PBST for 15 minutes, retinal sections were incubated in 5% goat serum for 30 minutes, and then, they were incubated with PBS containing the mouse 1D4 anti-rhodopsin antibody (20 μg/mL) for 2 hours at room temperature in a humidified chamber. The retinal sections were washed with PBST for four times and incubated with Cy3 conjugated goat anti-mouse antibody (5 μg/mL) for 1 hour at room temperature. Hoechst 33342 (1:10 000 dilution) was applied for 5 minutes to stain the nuclei. Sections were mounted with the ProlongGold mounting solution (ThermoFisher). Immunofluorescence images were then taken by a fluorescence microscope for low-magnification images and a confocal microscope for high-magnification images. A total of six high magnification images were taken using a 60× objective with oil per retinal cryosections at proximately 0.6, 1.0, and 1.4 mm to the ONH. Immunofluorescence intensity of rhodopsin in OS, and outer nuclear layers (ONL) were measured using ImageJ by selecting corresponding layers of rhodopsin in OS, and outer nuclear layers (ONL) were used Hek293 cells for HTS. Using this luciferase reporter available in culture, we selected one of the most commonly used P23H rhodopsin (Figure 1C). Together, 46 compounds with activity scores lower than the cutoff at mean-2SD were tested in one dose (9.96 to 16.13 μM) from three compound collections, and 128 molecules in a total of six compound collections (Figure 1B, Supplementary Table S1). Because a photoreceptor cell line is not a counter screen to select only those not reducing luminescence on the WT rhodopsin-expressing Hek293(RHOP23H-Rluc) cells (Hek293(RHOP23H-Rluc), Figure 1A and Supplementary Figure S1).33,34 Because a photoreceptor cell line is not available in culture, we selected one of the most commonly used Hek293 cells for HTS. Using this luciferase reporter assay to quantify P23H rhodopsin in response to 24 hours of compound treatment, we conducted an HTS of 68 979 small molecules in a total of six compound collections (Figure 1B, Supplementary Table S1). We identified 2072 compounds with activity scores lower than the cutoff at mean-2SD (Table I and Supplementary Data 1) tested at one dose (9.96 to 16.13 μM) from three compound collections, and 128 hits with EC50 < 20 μM tested at 7-11 doses from the other three compound collections (Supplementary Data 1). In the hit selection process, we excluded those compounds that inhibited the recombinant Rluc activity, and we performed a counter screen to select only those not reducing luminescence on the WT rhodopsin-expressing Hek293(RHOP23H-Rluc) cells at 10 μM compound concentrations, suggesting these selected compounds selectively favored the clearance of the mutant rhodopsin (Supplementary Data 1). Then, we tested the dose response effects of these compounds in the Hek293(RHOP23H-Rluc) and Hek293(RHOPWT-Rluc) cells by the luciferase reporter assay, to measure the potency and efficacy of these compounds that selectively reduced the P23H rhodopsin (Figure 1C). Together, 46 compounds with

3 | RESULTS

3.1 HTS Identified 46 compounds that selectively reduced P23H rhodopsin

To identify small molecules that clear the misfolded P23H rhodopsin mutant protein, we developed a cell-based HTS assay using a Hek293 cell line stably expressing the bright Rluc as a reporter fused to P23H rhodopsin (Hek293(RHOP23H-Rluc), Figure 1A and Supplementary Figure S1). Statistical analyses

The HTS and high-content imaging assays were performed with each plate containing 16 repeats of 0% and −100% controls and Z’ was calculated by plate to make sure Z’ > 0.5 for HTS and Z’ > 0 for high-content imaging assay indicating results in each plate were robust and activity scores calculated properly by the controls. Z’ = 1-3 × (SD0% control + SD−100% control)(Mean0% control−Mean−100% control).37

The ERG recordings were analyzed by a two-way analysis of variance (ANOVA),38 because the ERG responses can be affected by two factors, compound treatment (factor 1) and flash intensity (factor 2). p1 and p2 determined whether compound treatment and flash intensity significantly affect the ERG response, respectively; whereas p1-2 determined whether the two factors interact with each other. The other assays were analyzed by the unpaired two-tailed Student’s t test. The criteria for significance was: not significant, P > .05; *P < .05; **P < .01; ***P < .001; ****P < .0001. Sample size was chosen based on power analyses using the functions:

\[
1−\beta = \Phi \left( \frac{|\mu_1−\mu_2|\sqrt{n}}{\sigma_1+\sigma_2} − z_{1−\alpha} \right)
\]

where \( \sigma \) is SD, \( \Phi \) the standard normal distribution function, \( \alpha \) is Type I error or P value set at .05, \( \tau \) is the number of comparisons to be made, \( \beta \) is the Type II error, and 1-\( \beta \) is the power set at 0.90.39 Both males and females were included randomly in each sample group of the animal studies.
FIGURE 1  High-throughput screening (HTS) for small molecules that selectively reduce the misfolded P23H rhodopsin. A, Illustration of the cell-based luciferase reporter assay for HTS and counter screen. The mouse P23H (purple) or wild type (WT, green) rhodopsin was fused with Renilla luciferase (Rluc, yellow) and constitutively expressed in Hek293 cells, marked as the Hek293(RHOP23H-Rluc), or Hek293(RHOWT-Rluc), respectively. For the HTS, Hek293(RHOP23H-Rluc) cells were incubated with each compound for 24 hours before assayed for luciferase activity, and hits that showed activity scores lower than Mean-2SD were selected. For the counter screen, we tested the hits by repeating the luciferase reporter assay in the Hek293(RHOWT-Rluc) cells, and selected those that showed preferred clearance activity in the Hek293(RHOP23H-Rluc) vs the Hek293(RHOWT-Rluc) cells. B, A pie chart showing the compound libraries screened. The number of compounds in each library is shown in the bracket. UC, University of Cincinnati Diversity Set; LOPAC, Library of Pharmacologically Active Compounds; FDA, US Food and Drug Administration approved drugs; MIPE, NCATS Mechanism Interrogation Plat E. C, An exemplary dose-response plot of a hit compound, CL-009 in the Hek293(RHOP23H-Rluc) and Hek293(RHOWT-Rluc) cells, as black squares and magenta circles, respectively. The luminescence was normalized by the mean luminescence of cells treated with 0.1% DMSO and 1 mM Evans Blue as the 0 and −100% controls, respectively. Data points and error bars are the means and SDs. N = 3. Dose-response curves were fitted by the Origin Software using the modified Hill function. D, Rhodopsin dot blots of untreated NIH3T3(RHOWT/GFP) and NIH3T3(RHOP23H/GFP) cell lysates loaded at 25%, 50%, 75%, and 100%. E, Rhodopsin dot blot intensities in D were measured by ImageJ and plotted as a function of loaded cell lysate amount. N = 3. F, Rhodopsin dot blots of NIH3T3(RHOP23H/GFP) and NIH3T3(RHOWT/GFP) cells (bottom) each treated with 0.1% DMSO or CL-001 to CL-009 at 10 μM for 24 hours. Cells were loaded at the same amount as the 100% loading control in D (original scan of dot blots are shown in Supplementary Figure S2). G, Rhodopsin dot blot intensities in F were plotted in as a box chart, respectively. The middle lines and upper/bottom lines of boxes in G are the means and SDs. N = 3. P23H and WT rhodopsin levels in each repeat are shown as black squares and magenta circles, respectively. H, The chemical structures of CL-001 to CL-009. EC50s shown in brackets were obtained from high-content image analyses quantifying the immunostaining of P23H rhodopsin in response to 8-10 doses of each hit compound.
the mutant rhodopsin selectivity were confirmed from this HTS of 68 979 compounds.

3.2 Hit validation confirmed 9 hits

To eliminate the false positives related to cell lines or Rluc fusion and confirm the selective activity of 46 hits on clearance of P23H over WT rhodopsin, we tested these compounds in two NIH3T3 cell lines stably expressing the P23H rhodopsin (NIH3T3(RHOP23H/GFP)) and WT rhodopsin (NIH3T3(RHOWT/GFP)), respectively. We used a different cell line from the Hek293 that was used for HTS to select compounds with activities that are not cell type specific. We also determined the rhodopsin levels using the dot blots of cell lysates from NIH3T3(RHOP23H/GFP) and NIH3T3(RHOWT/GFP) cells treated with 10 μM of each compound that showed a selective decrease of P23H compared to WT rhodopsin (Figure 1D-G and Supplementary Figure S2). We used high-content imaging analysis of rhodopsin immunofluorescence to quantify P23H and WT rhodopsin in these cells after 24 hours treatment with these compounds (Figures 1H and 2). As a result, we validated nine compounds that selectively cleared P23H rhodopsin in mammalian cells (Figure 1H and Table 2).

3.3 Chemoinformatics of the 9 mutant rhodopsin selective hits

Among the nine hits, only two compounds (CL-002 and CL-004) have no previously known pharmacological activities. Four of them (CL-001, CL-006, CL-007, and CL-008) were pan-cyclin-dependent kinase inhibitors (Figure 1H). CL-003 has been documented with activities in numerous assays targeting different proteins including nucleotide binding oligomerization domain containing 1 (NOD1), NOD2, huntingtin, tumor necrosis factor α, glycogen synthase kinase 3, and so forth, suggesting its activity in inducing hypoxia responses. CL-009, MTX, is an approved drug for the treatment of cancer and rheumatoid arthritis. However, the molecular mechanisms of action by which these compounds mediate P23H rhodopsin clearance require further investigation.

3.4 Effect of nine confirmed compounds on rhodopsin transcription and biodegradation

We performed qPCR and a non-radioactive pulse-chase assay in NIH3T3(RHOP23H/GFP) and NIH3T3(RHOWT/GFP) cells treated with or without each hit compound for 24 hours to determine whether the effect of selective clearance of P23H rhodopsin is due to either reduced biosynthesis or increased degradation of rhodopsin. The qPCR showed that both WT and P23H rhodopsin transcripts were reduced non-selectively by CL-001, CL-002, CL-003, CL-004, CL-006, CL-007, and CL-008, in comparison to DMSO control (Figure 3A). Surprisingly, CL-005 increased rhodopsin transcripts in both NIH3T3(RHOP23H/GFP), and NIH3T3(RHOWT/GFP) cells up to two-fold compared to DMSO control, whereas CL-009/MTX did not affect the transcription of both the WT and P23H rhodopsin.

In the non-radioactive pulse-chase assay, we transiently labeled nascent proteins for 4 hours by replacing Met with AHA, an analogue of Met with an azide group in the side chain, in the culture medium of NIH3T3(RHOP23H/GFP) and NIH3T3(RHOWT/GFP) cells, followed by addition of Met back to the medium and chased for 24 hours. The remaining AHA labeled rhodopsin was measured by attaching BTN to AHA incorporated proteins via a “Click” reaction, immunoprecipitating the cell lysate with 1D4 anti-rhodopsin antibody and dot blotted with SA (Figure 3B). We found that the BTN-AHA labeled P23H rhodopsin was significantly reduced at 24 hours of chase by treatment with 10 μM of CL-001, CL-002, CL-005, CL-007, and CL-009 (MTX), in comparison to DMSO control (Figure 3C and Supplementary Figure S3). The total P23H rhodopsin pull-down level was decreased by all nine hits tested, confirming their previously validated activity (Supplementary Figure S3F). This result suggests only these five compounds (CL-001, CL-002, CL-005, CL-007, and CL-009/MTX) accelerated the degradation of the misfolded rhodopsin. Together, the results of rhodopsin dot blot, qPCR and pulse-chase assay showed that (Table 3) the hit compounds reduced the P23H rhodopsin by: (1) reducing rhodopsin transcription only (CL-003, CL-004, CL-006, and CL-008); (2) increasing its degradation only (CL-005 and CL-009); (3) or both decreasing transcription and increasing degradation of P23H rhodopsin (CL-001, CL-002, and CL-007). Because the transcription of rhodopsin in the stable cells is driven by the cytomegalovirus (CMV) promoter but not the rhodopsin promotor, thus the group 1) compounds were not included for further investigation in this study because they may not affect the P23H rhodopsin level in vivo. Only 5 compounds (CL-001, CL-002, CL-005, CL-007, and CL-009) remained for further study.

3.5 The activity of five compounds on the clearance of other misfolded rhodopsin mutants

To determine whether these five confirmed compounds affect the clearance of other RP-causing rhodopsin mutants in vitro, we measured the protein levels of six adRP-causing rhodopsin
The high-content imaging assays validated nine hits that selectively reduced the P23H rhodopsin in vitro. For hit confirmation, we used the NIH3T3 cells that stably co-expressed GFP and P23H or WT rhodopsin, marked as NIH3T3(RHO<sup>P23H/GFP</sup>) or NIH3T3(RHO<sup>WT/GFP</sup>), respectively. A, High-content images of cells treated with 0.1% DMSO or CL-001 to CL-009 at 10 μM for 24 hours. Immunostaining of rhodopsin (magenta) showed that CL-001 to CL-009 selectively reduced the P23H but not the WT rhodopsin level. Scale bar, 50 μm. B–J, Dose-response curves of nine hit compounds by image-based analysis. Relative immunostaining intensity of rhodopsin measured from high-content images of NIH3T3(RHOP23H/GFP) (black squares) and NIH3T3(RHW/GFP) cells (magenta circles) each treated with DMSO or CL-001 to CL-009 at 8-10 doses for 24 hours. The immunostaining intensity of rhodopsin per cell was normalized by DMSO-treated cells as 0% and cells stained with secondary antibody only as −100%, respectively. Data and error bars are means and SDs. N = 3. Dose-response curves were fitted by a modified Hill function. Chemical structure and EC<sub>50</sub> of each hit compound in the NIH3T3(RhoP23H/GFP) are shown in the inset of each graph.
**TABLE 2** Pharmacological activities of confirmed compounds that selectively clear P23H rhodopsin

| Hit name | PubChem CID | Libraries | Rluc assay Hek293 (RHO<sup>P23H</sup>-Rluc) | Rluc assay Hek293 (RHO<sup>WT</sup>-Rluc) | Image-based assay NIH3T3 (RHO<sup>P23H</sup>/GFP) | Image-based assay NIH3T3 (RHO<sup>WT</sup>/GFP) |
|----------|-------------|-----------|-------------------------------------------|-------------------------------------------|---------------------------------|---------------------------------|
|          |             |           | EC<sub>50</sub> (µM) | Efficacy (%) | EC<sub>50</sub> (µM) | Efficacy (%) | EC<sub>50</sub> (µM) | Efficacy (%) | EC<sub>50</sub> (µM) | Efficacy (%) |
| CL-001   | 11 715 767  | MIPE      | 1.1 | −69.4 | 1.0 | −44.3 | 2.1 | −83.6 | NA | NA |
| CL-002   | 6 224 422   | Life Chemicals | 2.0 | −86.1 | 3.4 | −62.0 | 1.6 | −61.0 | NA | NA |
| CL-003   | 4 438 424   | Life Chemicals | 0.89 | −65.3 | 1.5 | −37.7 | 0.60 | −46.1 | NA | NA |
| CL-004   | 6 624 030   | Life Chemicals | 1.6 | −68.6 | 0.92 | −45.5 | 0.47 | −70.7 | NA | NA |
| CL-005   | 10 091 681  | UC        | 6.6 | −78.2 | 4.2 | −57.9 | 1.3 | −72.7 | NA | NA |
| CL-006   | 11 338 033  | MIPE      | 1.7 | −66.2 | 1.52 | −42.9 | 2.2 | −76.4 | NA | NA |
| CL-007   | 5 330 790   | MIPE      | 13 | −72.5 | 11 | −51.7 | 2.9 | −72.7 | NA | NA |
| CL-008   | 16 747 683  | MIPE      | 0.93 | −89.5 | 0.59 | −52.7 | 1.6 | −70.1 | NA | NA |
| CL-009   | 126 941     | Spectrum  | 0.43 | −53.0 | NA | NA | 3.3 | −36.0 | NA | NA |

*Note:* The activity scores of each assay were normalized by the 0% and −100% controls. For the Rluc assay, DMSO-treated group was the 0% control, and 1 mM Evans Blue-treated group was the −100% control. For the image-based assay, the DMSO-treated group was the 0% control, and cells immunostained with secondary antibody only was the −100% control. Compounds labeled in bold were shown to increase Rho<sup>P23H</sup> degradation. MIPE, the NCATS Mechanism Interrogation Plat E; Life Chemicals, the Life Chemicals 50K Collection; UC, the university of Cincinnati 10K Diversity Set; and Spectrum, the Spectrum Collection. NA, not applicable because the compound showed no dose response effect.
FIGURE 3  Effect of active compounds on rhodopsin transcription, degradation, and clearance of other adRP causing mutants. A, Fold change of RHO transcripts in the NIH3T3(RHO<sup>P23H</sup>/GFP) and NIH3T3(RHO<sup>WT</sup>/GFP) cells treated with 10 μM of each hit compound compared to DMSO control. Q-PCR result of RHO transcript was first normalized by β-actin, and then, by the DMSO control. Middle lines and error bars are means and SDs of three biological replicates shown as data points. N = 3. RHOP23H, black squares; and RHOWT, magenta circles. B, Illustration of the non-radioactive pulse-chase assay. Briefly, cells were starved in a Met-free medium for 1 hour before pulsed in the azidohomoalanine (AHA) enriched medium that lacks Met for 4 hours, so the nascent protein synthesized was labeled with AHA. Cells were then chased for 0-24 hours in the medium containing 2 mM of Met, so the protein synthesized during the chase period was not labeled with AHA anymore. Next, via a “Click” reaction, the AHA incorporated proteins in the cell lysate were linked with biotin (BTN). Total rhodopsin was immunoprecipitated (IP) by the 1D4 anti-rhodopsin antibody, and the BTN labeled rhodopsin was finally dot blotted (IB) with HRP-Streptavidin (SA). C, Percentage of nascent P23H rhodopsin from NIH3T3(RHOP23H/GFP) cell lysates IP with 1D4 anti-rhodopsin antibody (RHO) and IB with SA at 24 hours of chase time. Cells were treated with 10 μM of CL-001 to CL-009, or DMSO at 0 hours of chase time, respectively. The IB intensity of each dot was normalized by the DMSO control in the same membrane (see Supplementary Figure S3). Three lines in each box represent the 75%, 50%, and 25% values of data in each group, and mean of each group was shown as filled diamonds, error bars were SDs. N = 3. *P < .05; **P < .01; and ***P < .001 by an unpaired two-tail Student’s t test. D, Immunostaining images of rhodopsin in U2OS cells stably expressing the WT or six mutants of mouse rhodopsin (T4R, P23H, P53R, C110Y, D190N, and P267L) that cause autosomal dominant retinitis pigmentosa, under treatment with 10 μM of CL-001, CL-002, CL-005 (11 μM), CL-007, and CL-009. Scale bar, 100 μm
mutants stably expressed in the U2OS cells (T4R, P23H, P53R, C110Y, D190N, and P237L, Supplementary Figure S1) when they were treated with these compounds. These Class II mutants were previously reported to cause rhodopsin misfolding (www.hgmd.cf.ac.uk). The U2OS cells used here were previously used to quantify the effect of small molecule chaperones on rescuing rhodopsin transport.24 Using immunofluorescence and high-content imaging to quantify rhodopsin levels, we found that none of the five compounds affected the cellular localization of rhodopsin mutants, but the immunofluorescence intensities of these rhodopsin mutants were reduced by CL-001, CL-002, CL-005, and CL-007 (Figure 3D and Supplementary Figure S4). CL-009 (MTX) treatment only showed dose-dependent reductions in cells expressing the P23H, C110Y, D190N, and P267L mutants, but not the T4R and P53R mutants. The slightly different pharmacological activity of these compounds on P23H rhodopsin clearance in NIH3T3 (Figure 2A) and U2OS cells (Figure 3D) is due to the difference of cell type and expression level of P23H rhodopsin in the two stable cell lines. We then focused on CL-009 (MTX) for its mechanism of action and in vivo efficacy studies because it is the only compound that accelerates mutant rhodopsin degradation without an effect on its transcription.

3.6 | MTX mediated P23H rhodopsin clearance via the lysosome but not proteasome pathway

Rhodopsin is degraded via both the proteasome and the lysosome pathways.42,43 To determine which proteolytic pathway is involved in MTX mediated P23H rhodopsin clearance, we treated the NIH3T3(RHO<sup>P23H</sup>/GFP) and NIH3T3(RHO<sup>WT</sup>/GFP) cells with MTX plus either BafA1, an ATPase inhibitor preventing lysosome acidification and activity, or MG-132, a proteasome inhibitor, and quantified the P23H or WT rhodopsin levels in these cells by immunoblots. We found only BafA1 but not MG-132 treatment abolished MTX-induced P23H rhodopsin clearance, suggesting the lysosome rather than the proteasome pathway was involved in the MTX mediated P23H rhodopsin clearance (Figure 4A, B and Supplementary Figure S5). BafA1 and MTX co-treatment also led to an accumulation of WT rhodopsin, whereas MG-132 plus MTX did not (Figure 4A,B), suggesting both WT and P23H rhodopsin are degraded mainly via the lysosome pathway in these NIH3T3 stable cells.

We repeated these treatments and quantified rhodopsin levels by immunofluorescence and high-content imaging (Figure 4C,D). We found that the mean intensity of P23H rhodopsin immunostaining per cell was reduced in an MTX dose-dependent manner, which was not affected by the addition of MG-132 but was entirely abolished by co-treatment with BafA1. This result confirmed the above immunoblots data that MTX selectively improved the degradation of P23H rhodopsin via the lysosome pathway.

We further found that MTX treatment did not affect chymotrypsin-like proteasome activity in the NIH3T3(RHO<sup>P23H</sup>/GFP) and NIH3T3(RHO<sup>WT</sup>/GFP) cells (Figure 4E-G), confirming the conclusion that MTX did not affect the proteasome pathway.

3.7 | MTX increased autophagy flux in vivo

Because autophagy is known to regulate the clearance of protein aggregates via lysosomal activity, we then asked whether MTX treatment affects autophagy in Rho<sup>P23H/+</sup> knock-in mice, a commonly used animal model of RP.17,32,44 We administered 25 pmol of MTX by an IVI to one eye at PND 15 and an equal volume of PBS as vehicle control in the other eye. To determine whether autophagy flux was affected by MTX, we immunoblotted the LC3 and SQSTM1/p62 in the retinae after 48 hours

| Compounds name | Rho protein level (Dot blot) | Rho mRNA level (qPCR) | Rate of Rho<sup>P23H</sup> degradation (Pulse-chase) |
|----------------|-----------------------------|-----------------------|---------------------------------------------|
| CL-001         | Down                        | Down                  | Up                                          |
| CL-002         | Down                        | Down                  | Up                                          |
| CL-003         | Down                        | Down                  | No Change                                   |
| CL-004         | Down                        | Down                  | No Change                                   |
| CL-005         | Down                        | Up                    | Up                                          |
| CL-006         | Down                        | Down                  | No Change                                   |
| CL-007         | Down                        | Down                  | Up                                          |
| CL-008         | Down                        | Down                  | No Change                                   |
| CL-009         | Down                        | No Change             | Up                                          |

Note: The effect of nine hit compounds to NIH3T3(RHO<sup>P23H</sup>/GFP) cells compared to DMSO control. Down, decreasing; up, increasing; no change, no significant change. P < .05.

TABLE 3 Effect of nine confirmed compounds on transcription and degradation of rhodopsin
of treatments (Figure 5). LC3-II is the lipidated form of LC3 that is incorporated in the autophagosomes, and SQSTM1/p62 is a known cargo of autophagy flux.\textsuperscript{45} Compared to PBS control, MTX treatment led to a decrease of SQSTM1/p62 level (Figure 5B) and an increase of LC3-II (Figure 5C), suggesting MTX increased autophagy flux in vivo.

### 3.8 One IVI of MTX increased ERG response and retinal rhodopsin level in the Rho\textsuperscript{P23H/+} mice

Photoreceptors of the Rho\textsuperscript{P23H/+} mice undergo a period of fast degeneration from PND 15 to 1 month of age, and they die at a
slower rate afterward. We tested the effect of MTX treatment on the retinal function and structure during the fast phase of retinal degeneration in the RhoP23H/+ mice. We administered 25 or 100 pmol of MTX to one eye of these mice at PND 15 by an IVI and an equal volume of PBS as vehicle control in the other eye, and recorded scotopic and photopic full-field ERGs of these mice at PND 32. These two doses were estimated based on the volume of the mouse eyeball, and efficacious concentration of MTX in vitro. Exemplary scotopic ERG responses at 10 cd·s/m² showed that the a- and b-waves of 25 pmol MTX-treated RhoP23H/+ mouse eyes were higher than the PBS and non-treated groups (Figure 6A). Multi-flash scotopic ERG measurements confirmed that both a- and b-waves of 25 pmol MTX-treated RhoP23H/+ eyes were significantly higher than the PBS and non-treated groups (Figure 6A). Multi-flash scotopic ERG measurements confirmed that both a- and b-waves of 25 pmol MTX-treated RhoP23H/+ eyes were significantly higher than the PBS and non-treated groups, whereas 100 pmol MTX-treated eyes showed no effect (Figure 6B,C). The 25 pmol MTX-treated RhoP23H/+ eyes also showed higher photopic ERG responses than PBS treated or non-treated eyes (Figure 6D), but the 100 pmol of MTX did not show any effects. The ratio of scotopic and photopic b- to a-wave amplitudes was not affected by MTX treatment, suggesting the functional increase of b waves by 25 pmol MTX was mainly due to the increased photoreceptor function, but not the independently increased bipolar cell responses (Supplementary Figure S6A,B). The PBS-treated group showed no difference in scotopic or photopic responses to the non-treated group, suggesting that a single IVI was safe and did not affect visual function.

To examine the retinal structure and rhodopsin homeostasis, we immunostained the retinal cryosection from these treated mice (euthanized at PND 33) with the anti-rhodopsin antibody labeling the OS, and Hoechst33342 for nucleus staining. As previously reported, the non-treated RhoP23H/+ retina at PND 33 showed significantly shorter OS layer, reduced rhodopsin level and about half of nucleus number in the ONL compared to Rho+/+ retina, supporting that rhodopsin homeostasis was disrupted and retinal degeneration occurred in the RhoP23H/+ mice at 1 month of age (Figure 6E-J,Q,R and Supplementary Figures S6C,D and S7). The 25 pmol MTX-treated RhoP23H/+ retina showed a significant increase in the total rhodopsin level and rhodopsin in the OS, compared to PBS control on the superior side, but not the inferior side (Figure 6K-Q, and Supplementary Figures S6C,D and S7). No change in ONL nucleus number was seen on either side of the RhoP23H/+ retina by treatment of MTX (Figure 6R), suggesting one IVI of 25 pmol MTX may not be sufficient to protect the RhoP23H/+ mice from the fast period of retinal degeneration. PBS-treated retinas showed no difference in total rhodopsin level or localization, nor the ONL nucleus number, compared to untreated RhoP23H/+ retina, confirming that one IVI itself is safe and does not change retinal morphology (Figure 6H-M,Q,R and Supplementary Figure 6C,D). Combining the ERG and IHC results, we conclude that a single IVI of 25 pmol MTX improved ERG responses in the RhoP23H/+ eyes by increasing the functional rhodopsin level on the superior side of the retina that was not due to increased number of rod photoreceptors.

### 3.9 Multiple IVIs of MTX increased ERG response and rhodopsin level as well as photoreceptor cell numbers in the RhoP23H/+ mice

We then asked whether multiple administrations of MTX improved its efficacy in restoring rhodopsin homeostasis and preserving photoreceptors in the RhoP23H/+ mice (Figure 7). Thus, we performed four weekly IVIs of MTX to the RhoP23H/+ mouse eyes starting at PND 15, followed by ERG recordings at PND 44 and euthanasia of the animals at PND 46 for IHC. The multi-flash scotopic and photopic b-waves of the MTX-treated eyes were significantly increased compared to PBS control group (Figure 7A-D). Photopic b-waves of 100 pmol MTX-treated RhoP23H/+ eyes were also
higher than the PBS group at higher flash intensities, even though they were not as high as the 25 pmol MTX group (Figure 7D). The ERG recordings showed that multiple IVIs of MTX improved visual function compared to vehicle control. However, weekly IVIs of the vehicle showed reduction of both scotopic and photopic responses compared to untreated control (Figure 7A-D), suggesting the weekly IVIs compromised the visual function of Rho<sup>P23H/+</sup> mice.
FIGURE 6 One IVI of MTX increased electroretinogram (ERG) response and retinal rhodopsin level in RhoP23H/+ mice. Eyes of mice were untreated or intravitreally injected with PBS, 25 or 100 pmol MTX at PND 15, and ERG responses were recorded at PND 32. Mice were euthanized and eyes were enucleated at PND 33 for immunohistochemistry (IHC). Age-matched RhoP23H/+ mice were used as the normal control. A, Scotopic ERG recordings stimulated by a flash of light at 10 cd·s/m². B and C, Eight-flash scotopic a- and b-wave amplitudes of treated mice plotted as a function of flash intensity (semi-log format), respectively. D, Six-flash photopic b-wave amplitudes plotted as a function of flash intensity (semi-log format). Black squares, red circles, blue triangles, and magenta reverse triangles were from RhoP23H/+ mice that were untreated, PBS, 25 and 100 pmol MTX treated, respectively. Data points and error bars are means and SEMs, respectively. N = 5. *P < .05 between 25 pmol MTX and PBS treated groups calculated by a two-way ANOVA. Factor 1, treatment; and factor 2, flash intensity. E-P, IHC images of untreated RhoP23H/+ retina, and RhoP23H/+ mouse retinae that are untreated, PBS-treated, or 25 pmol MTX-treated, from top to bottom, respectively. RHO and nucleus (Hoechst 33342) were stained in red and blue, respectively. E, H, K, and N are retinal IHC images at low magnification. Scale bar, 500 μm. F, I, L, and O are high magnification retinal images taken at sites marked in boxes shown in E, H, K, and N on the retinal inferior side, and G, J, M, and P are images on the superior side, respectively. Scale bar, 50 μm. Q, Spidergram of rhodopsin immunofluorescence intensity in the OS measured by ImageJ from high magnification images taken at 0.6, 1, and 1.4 mm from the optic nerve head (ONH). Green dimonds, age-matched RhoP23H/+ mouse retinae. R. Spidergram of outer nuclear layer (ONL) nucleus number per 200 μm length of retina cross-section images taken at 0.6, 1, and 1.4 mm distance from ONH. Data points and error bars are means and SEMs, respectively. N = 3. *P < .05 between 25 pmol MTX and PBS groups by an unpaired two-tail Student’s t test

The IHC of RhoP23H/+ retinae treated with four IVIs of 25 pmol MTX showed significantly higher levels of rhodopsin in the OS, lower level of rhodopsin in the ONL, and higher number of nucleus number in the ONL, on the superior side, but not the inferior side, in comparison to the PBS group (Figure 7E-O and Supplementary Figures S6E,F and S8). The results suggested that four IVIs of 25 pmol MTX increased folded rhodopsin in the OS and decreased mislocalized rhodopsin in the ONL on the superior side of the retina, even though we cannot distinguish the WT rhodopsin from the mutant. Compared to one injection of 25 pmol MTX, we found four weekly IVIs of MTX showed higher efficacy in retina protection, which preserved more ONL nucleus number on the superior side compared to the PBS group, but one MTX injection did not. However, four weekly IVIs of PBS showed reduced total rhodopsin level and lower nucleus number in the ONL of the RhoP23H/+ retinae, compared to untreated control, confirming an adverse side effect by the multiple weekly IVIs that is also seen in the ERG responses (Figure 7E-J,N-O). Future optimization of IVI intervals or change in the treatment route is needed for long-term MTX treatment.

4 | DISCUSSION

Therapeutic strategies for RP at different stages are different, depending on the surviving photoreceptor number at the time of intervention. To restore vision for the late-stage RP, many efforts have been devoted to varieties of techniques including stem cell therapies, optogenetics, and retina prosthetic developments to re-build visual responses in the retinae where most photoreceptors are gone. Gene therapy has made a substantial breakthrough for treating autosomal recessive blindness mainly by delivery of the functional gene locally that is lost due to genetic mutations when retinal structures are still largely maintained. Alternatively, gene delivery of neurotrophic factors by adeno-associated virus such as ciliary neurotrophic factor and cone-rod derived neurotrophic factor showed protective effects that delayed rod and cone death in animal models of RP, respectively. In complementation to gene therapy, we are looking for pharmacological interventions targeting the early events in the rods before they die, so that the retinal structure and function can be preserved, and vision can be maintained at the early- or mid-stages of RP. Specifically, we are targeting adRP, where rod death is not due to insufficiency of a gene’s function, but rather the dominant-negative effect of the mutated gene, such as RHO. Thus, the goal of this study is to develop preventive therapies targeting the early- and mid-stages of adRP caused by misfolded rhodopsin. Importantly, we discovered a novel activity of MTX, an FDA-approved drug, that upregulated misfolded rhodopsin degradation and improved visual function, preserved retinal structure from the fast period of retinal degeneration in the animal model of RP. Potentially, this misfolded protein degradation pathway upregulated by MTX may not be restricted to the clearance of rhodopsin alone that could be applied to other misfolded protein-associated blindness such as myocilin-associated primary open-angle glaucoma.

The molecular pathways regulating the protein homeostasis of G protein coupled receptors are not well understood. By screening both novel and pharmacologically active small molecule compounds, we were able to explore the chemical genetics and find out the most relevant molecular pathways that regulate rhodopsin homeostasis. Here, we identified five compounds that increased misfolded rhodopsin degradation on the HTS campaign of small molecules: CL-001 and CL-007 are pan-cyclin-dependent kinase inhibitors; CL-005 is a stabilizer of HIF1α; CL-009 (MTX) is an inhibitor of folic acid metabolism; and only CL-002 is an unknown chemical without reported pharmacological activities. Although this study only focused on MTX for the mechanisms of action and in vivo effect, exploring potential roles of CKDs or other...
kinases, as well as modulators of HIF1α in mediating misfolded rhodopsin degradation are exciting future directions that may lead to a better understanding of membrane protein degradation.

MTX is water-soluble and it has been intravitreally administered as an off-tag treatment for inflammatory ocular diseases such as retina uveitis. Thus, the novel activity of MTX in the selective clearance of the P23H rhodopsin and its

FIGURE 7 Multiple IVIs of MTX improved ERG response, rhodopsin level, and preserved photoreceptor cell numbers of the RhoP23H/+ mouse retinas. Eyes of RhoP23H/+ mice were untreated or administered by four weekly IVIs of PBS, 25 pmol MTX and 100 pmol MTX per treatment, starting at PND 15 and ERGs were taken at PND 44. Eyes were enucleated at PND 46 for IHC. A, Scotopic ERG responses stimulated by a flash of light at 10 cd·s/m². B and C, Eight-flash scotopic a- and b-wave amplitudes of treated mice plotted as a function of flash intensity (semi-log format), respectively. D, Six-flash photopic b-wave amplitudes plotted as a function of flash intensity. Black squares, red circles, blue triangles, and magenta reverse triangles are from RhoP23H/+ mice that were untreated, PBS, 25 and 100 pmol MTX treated, respectively. Data points and error bars are means and SEMs, respectively. N = 5. *P < .05 between 25 pmol MTX and PBS-treated groups calculated by a two-way ANOVA. Factor 1, treatment; and factor 2, flash intensity. E-M, IHC images of untreated, PBS-treated, and 25 pmol MTX-treated RhoP23H/+ mouse retinas, from top to bottom, respectively. RHO and nucleus (Hoechst 33342) were stained in red and blue, respectively. E, H, and K are retinal images at lower magnification. Scale bar, 500 μm. F, I, and L are high magnification retinal images taken at sites marked as boxes shown in E, H, and K on the inferior side, and in G, J, and M are images on the superior side, respectively. Scale bar, 50 μm. N, Spidergram of rhodopsin immunofluorescence intensity in the OS measured by ImageJ from high magnification immunofluorescence images taken at 0.6, 1, and 1.4 mm from ONH. O, Spidergram of ONL nucleus number per 200 μm length of retina cross-section images taken at 0.6, 1, and 1.4 mm from ONH. Data points and error bars are means and SEMs, respectively. N = 3. *P < .05 between 25 pmol MTX and PBS groups by an unpaired two-tail Student’s t test.
MTX showed clear in vitro activity in the selective clearance of the P23H rhodopsin. Thus, it is counter-intuitive to observe an increase of rhodopsin level on the superior side of the Rho\textsuperscript{P23H/+} mouse retina after a single or multiple IVIs of MTX. Most of the rhodopsin immunostain is on the OS of MTX-treated Rho\textsuperscript{P23H/+} mouse retinae (Figures 6 and 7), suggesting that the increased rhodopsin by MTX is adequately folded and transported to the targeted site. Considering the heterozygous background of the knock-in mice we tested here and that anti-rhodopsin antibody cannot distinguish P23H from WT rhodopsin, MTX’s activity in increasing the folded rhodopsin level in the OS can be due to its selective clearance of P23H rhodopsin in vivo and less co-aggregation of WT rhodopsin with the mutant rhodopsin in the ER. Indeed, we saw an increased autophagy flux after an IVI of MTX in the Rho\textsuperscript{P23H/+} mice, suggesting MTX may enhanced the misfolded rhodopsin degradation via inducing autophagy. To test this postulation, we determined the effect of MTX treatment on P23H rhodopsin level in the Rho\textsuperscript{P23H/P23H} mice by an IVI of 25 pmol MTX at PND 15 followed by retinal immunoblotting at 24, 48, and 72 hours after treatment. However, due to the low protein level of P23H rhodopsin in the Rho\textsuperscript{P23H/P23H} mice (about 1/200 of rhodopsin compared to the Rho\textsuperscript{+/+} retina), and high variations between individual animals, we did not see consistently and statistically significant difference of P23H rhodopsin by treatment of MTX, compared to PBS control (data not shown).

Spatial difference in retinal degeneration is known in the animal models of RP\textsuperscript{D7} as well as in RP patients,\textsuperscript{67,68} but we are not clear why the inferior side of the retina degenerate faster than the superior side in RP. Interestingly, we observed repetitive asymmetric efficacy of MTX treatment to the Rho\textsuperscript{P23H/+} retinae only on the superior side. This spatial difference in response to drug treatment has also been seen in IVI of neurotrophic factors to a rodent model of RP.\textsuperscript{69} Differential gene expression or light exposure between the superior and inferior retinae could contribute to the spatial susceptibility of MTX treatment.

The reason for MTX showed better retinal protection at 25 pmol than 100 pmol requires further investigation. One potential explanation could be the cytotoxicity of MTX at higher dose that may counter act with its protective effect by clearing out misfolded rhodopsin. Retinal damages were observed in rabbits with intravitreal injection of 1.76 µmol MTX (about 1 mM final concentration in vitreous, considering rabbit’s vitreous cavity is about 1.5 mL).\textsuperscript{70} Mouse vitreous volume is about 5.3 µL, thus 100 pmol of MTX will yield an initial vitreous concentration of 18.9 µM, whereas 25 pmol of MTX administration gave about 4.7 µM vitreous concentration (EC\textsubscript{50} of MTX is about 3.3 µM in vitro). Even though we did not see obvious retinal degeneration caused by 100 pmol MTX, this result indicates that a thorough toxicity study of MTX is required for our future study. A transcriptome analysis by RNA-Seq will also be an important future direction to understand the molecular pathways altered by MTX.

Caution should be taken with multiple IVIs, because our results demonstrate that four weekly IVIs of sterile PBS accelerated retinal degeneration in the Rho\textsuperscript{P23H/+} mice compared to the untreated control. However, the four-dose MTX-treated retinae showed increased photoreceptor numbers compared to PBS control, whereas one injection of MTX did not have this effect, suggesting one injection is not sufficient for long-term retinal protection in RP. A systemic administration of MTX is not ideal for treating RP because this compound is water-soluble and may not be able to cross the retina-blood barrier. A high dose of MTX can cause severe side effects that are not clinically applicable. A future development of optimized IVI intervals or a slow-release formula is required for long-term treatment with MTX to avoid adverse effects by frequent IVIs.

Our study, as far as we know, is the first thorough small molecule screening looking for compounds that accelerate misfolded rhodopsin degradation. We have discovered five compounds that selectively accelerated misfolded rhodopsin degradation in vitro. Future studies to identify their specific molecular targets and mechanisms of action will improve our current understanding of cellular regulation of rhodopsin homeostasis. Our proof-of-concept study showed that MTX, by selectively increasing the misfolded P23H rhodopsin degradation, improved the overall rhodopsin homeostasis, and preserved visual function and retinal structure in the Rho\textsuperscript{P23H/+} knock-in mouse model.

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AUTHOR CONTRIBUTIONS

Y. Chen designed the research; X. Liu, B. Feng, A. Vats, H. Tang, M. Swaroop, and Y. Chen performed research; X. Liu, B. Feng, H. Tang, M. Swaroop, G. Tawa, and Y. Chen
analyzed data; L. Byrne, W. Zheng and M. Schurdak provided technical and facility supports; and X. Liu, W. Zheng, M. Schurdak and Y. Chen wrote the paper.

CONFLICT OF INTEREST
The authors have no conflict of interests.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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