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Emixustat is a visual cycle modulator that has entered clinical trials as a treatment for age-related macular degeneration (AMD). This molecule has been proposed to inhibit the visual cycle isomerase RPE65, thereby slowing regeneration of 11-cis-retinal and reducing production of retinaldehyde condensation byproducts that may be involved in AMD pathology. Previously, we reported that all-trans-retinal (atRAL) is directly cytotoxic and that certain primary amine compounds that transiently sequester atRAL via Schiff base formation ameliorate retinal degeneration. Here, we have shown that emixustat stereoselectively inhibits RPE65 by direct active site binding. However, we detected the presence of emixustat-atRAL Schiff base conjugates, indicating that emixustat also acts as a retinal scavenger, which may contribute to its therapeutic effects. Using agents that lack either RPE65 inhibitory activity or the capacity to sequester atRAL, we assessed the relative importance of these 2 modes of action in protection against retinal phototoxicity in mice. The atRAL sequestrant QEA-B-001-NH2 conferred protection against phototoxicity without inhibiting RPE65, whereas an emixustat derivative incapable of atRAL sequestration was minimally protective, despite direct inhibition of RPE65. These data indicate that atRAL sequestration is an essential mechanism underlying the protective effects of emixustat and related compounds against retinal phototoxicity. Moreover, atRAL sequestration should be considered in the design of next-generation visual cycle modulators.

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Introduction

Maintenance of photoreceptor light sensitivity and health critically depends on the provision of a constant supply of the visual chromophore 11-cis-retinal (1–4). This demand is met by a metabolic pathway known as the visual cycle that is composed of enzymes and retinoid-binding proteins expressed in the photoreceptors and the adjacent retinal pigment epithelium (RPE). An important first step in this series of reactions is the reduction of all-trans-retinal (atRAL) to all-trans-retinol (atROL), which serves to both clear the reactive retinaldehyde released from photoactivated visual pigments and enable the subsequent trans-cis isomerization step of the pathway (5). Certain environmental insults including prolonged exposure to intense light, in combination with an unfavorable genetic background, can overwhelm the retinal reductase capacity of the visual cycle, leading to pathological side reactions of retinaldehyde that ultimately disrupt retinal structure and function (6, 7). A clinical example is an inherited juvenile macular degeneration known as Stargardt disease, in which mutations in the photoreceptor-specific ATP-binding cassette transporter (ABCA4) result in delayed atRAL clearance (8, 9). The resulting increased concentrations of atRAL exert a direct cytotoxic effect on photoreceptors (10), in addition to contributing to the formation of side-products such as N-retinylidene-N-retinylethanolamine (A2E) and retinal dimer (11–13).

The focus on A2E as a primary toxin (14, 15), despite some evidence to the contrary (16, 17), led to the belief that inhibiting the visual cycle could have beneficial effects on averting light-induced retinal damage in certain forms of retinal and macular degeneration by lowering the density of visual pigments. This concept was first tested in patients treated with 13-cis-retinoic acid (isotretinoin) or its derivatives (18–20) and led to the development of an inhibitor of RPE65, the non-retinoid derivative of retinylamine (Ret-NH2) emixustat (previously known as ACU-4429) (21). Currently, emixustat hydrochloride is being tested in a phase IIb/III multicenter, randomized, double-masked, dose-ranging study by comparing its efficacy and safety with placebo for the treatment of dry age-related macular degeneration (AMD) (ClinicalTrials.gov identifier: NCT01802866) (21). Oral administration of this compound in humans results in dose-dependent suppression of the scotopic electroretinogram (ERG) b-wave, which is a proposed surrogate marker of its efficacy in treating dry AMD (21, 22). However, an extension of this presumed therapeutic effect is a debilitating inhibition of dark adaptation resulting in night blindness, an adverse effect also observed in patients treated with...
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The photoreceptor atRAL concentration can reach 5 mM if all rhodopsin is bleached (4, 27). In vitro, atRAL is cytotoxic, even at low micromolar concentrations (28–30). The atRAL released during photobleaching is primarily toxic to photoreceptor cells rather than to the RPE, as shown by 2-photon microscopy (TPM) (31). We demonstrated that the retina was spared from light-induced degeneration by trapping the excess of this aldehyde with Ret-NH₂, and thus its mechanism of action could be more complex than noted in the limited evaluations reported to date.

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Figure 1. Inhibition of RPE65 by retinol analogs and formation of emixustat amide. (A) Primary amines used in this study that are structurally similar to retinol (vitamin A). (B) 11-cis-Retinal production in the presence of inhibitors depicted in A. Primary amines were preincubated with bovine RPE microsomes at room temperature for 5 minutes, then all-trans-retinal was added and the mixture incubated at 37°C. All incubation mixtures were quenched by the addition of methanol after 1 hour of incubation. Inhibition of RPE65 enzymatic activity was measured as a decline in 11-cis-retinal production. Note that (S)-emixustat was 10 times more potent than Ret-NH₂, and in lowering 11-cis-retinal production. Without an inhibitor, typical activity was between 25 and 32 pmol/minute, and this was set as 100% activity. (C) Extracted ion LC-MS chromatograms showing acylation of emixustat (m/z = 264.2 [MH]+; lower trace) by LRAT in RPE microsomes to form corresponding emixustat palmamidade (m/z = 502.3 [MH]+; upper trace).

Complete elucidation of the molecular pharmacodynamics of emixustat is highly relevant to the continued development of visual cycle modulators as therapeutic agents for treatment of retinal disease. The structural similarity between Ret-NH₂ and emixustat suggests that these agents could exert their protective effects through similar modes of action. In this study, we used mouse models to investigate the potency of RPE65 inhibition by emixustat, its utilization by lecithin:retinol acyltransferase (LRAT), the pharmacological selectivity of its 2 enantiomeric forms, its retention in the eye, and its ability to protect against acute light-induced retinal degeneration. Using a chemical biology approach informed by crystal structures of RPE65 in complex with emixustat, we assessed the relative importance of RPE65 inhibition and atRAL scavenging by visual cycle modulators in the protection against retinal phototoxicity in mice.

Results

Inhibitory effects of Ret-NH₂ and emixustat in biochemical assays. Ret-NH₂, the first described potent inhibitor of RPE65 (33), 2 enantiomers of emixustat, and QEA-B-001-NH₂ (34) (Figure 1A and supplemental material; available online with this article; doi:10.1172/JCI80950DS1) were tested for their ability to inhibit the RPE65-dependent retinoid isomerization reaction in a dose-dependent manner (Figure 1B). Amines were incubated with bovine RPE microsomes in the presence of atROL and the 11-cis-retinoid-binding protein CRALBP. The decrease in 11-cis-retinal production reflects inhibition of RPE65 by a tested amine. The compounds had the following IC₅₀ values: Ret-NH₂, 2.03 ± 0.39 μM; (S)-emixustat, 150 ± 24 nM; and (R)-emixustat, 91 ± 6 nM. QEA-B-001-NH₂ failed to prevent 11-cis-retinal production significantly at tested concentrations as previously reported (Figure 1B) (34). We did not detect any racemization of emixustat in RPE membranes or any significant binding to CRALBP.

Retinoid-like primary amines are acylated by the enzymatic action of LRAT (35). The resulting fatty acid amides of Ret-NH₂, other inhibitors of the visual cycle (18–20). 11-cis-Retinal acts as an opsin inverse agonist (23). Therefore, suppression of 11-cis-retinal production by visual cycle inhibitors gives rise to unliganded, constitutively active opsins that initiate low-level activation of the phototransduction cascade (ref. 24 and references therein). Over time, this chronic signaling results in photoreceptor degeneration, as observed in patients with type 2 Leber congenital amaurosis (25), in Rpe65-KO mice (24), and in mice chronically treated with visual cycle inhibitors (26). Dyschromatopsia, attributed to aberrant rod effects on cone signaling pathways, was also frequently reported by patients treated with emixustat (21, 22). Despite lacking a retinoid backbone, emixustat structurally resembles retinol/Ret-NH₂, and thus its mechanism of action could be more complex than noted in the limited evaluations reported to date.

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are storage forms of the compounds, which are then slowly hydrolyzed to evoke long-lasting suppression of retinoid isomerase activity (reviewed in refs. 36 and 37). Similar to Ret-NH₂, emixustat was also efficiently amidated upon incubation with bovine RPE microsomes (Figure 1C), indicating that tissue uptake of the drug can be facilitated by LRAT enzymatic activity.

The structure of emixustat bound to RPE65. To gain insights into the molecular mode of RPE65 inhibition, we determined crystal structures of RPE65 in complex with emixustat. Structures were obtained in the presence of racemic emixustat as well as its pure enantiomers. We observed an unambiguous residual active site electron density corresponding to the bound inhibitor in all 3 cases (Supplemental Figure 1). Residual maps also indicated the presence of bound palmitate in the adjacent pocket of the active site, with its carboxylate oxygen forming a monodentate coordinate bond with the active site iron (Supplemental Figure 1). The hydroxyl moiety of emixustat was hydrogen bonded to the hydroxyl group of Thr147, whereas the primary amine was involved in polar interactions with the carboxylate groups of Glu148 and the bound palmitate ligand. Crystals obtained in the presence of racemic emixustat exhibited electron density consistent with an exclusive binding of the (R)-isomer, which consistent with the emixustat-bound RPE65 structure obtained in an alternative space group (38). The stereochemical assignment was further confirmed by structures obtained in the presence of pure (R)- and (S)-emixustat, which revealed distinctly oriented electron densities for the hydroxyl moieties of these compounds (Figure 2). Density for the 3-amino-1-phenylpropan-1-ol moiety of (R)-emixustat was highly similar to that observed for the structure obtained from the racemic compound (Figure 2). These findings indicate that (R)-emixustat has higher binding affinity for RPE65 compared with that of the (S)-isomer, consistent with the slightly greater potency of the (R)-isomer compared with that of the (S)-isomer in the retinoid isomerase inhibition assay. The difference in binding affinity can be at least partially explained by the less favorable polar interaction we observed for the (S)-isomer compared with that for the (R)-isomer. Bond lengths for the hydroxyl-Thr147 Oγ, amine-Glu148 Oε2, and amine-palmitate O1 interactions were 3.1 Å, 3.2 Å, and 2.8 Å for the (S)-isomer compared with the 3 Å, 2.7 Å, and 2.6 Å distances observed for the (R)-isomer, respectively. The difference in affinity was also evident in the disproportionately higher average B factor for (S)-emixustat compared with that for (R)-emixustat, which could indicate either looser ligand binding or lower occupancy (Table 1).

In contrast to the polar end of the emixustat molecule, its cyclohexyl moiety exhibited structural flexibility, as evidenced by its higher atomic B factor and its ability to adopt distinct conformations in different crystal forms (Supplemental Figure 2). In the structure obtained from racemic emixustat, the planes of the cyclohexyl and phenyl moieties were approximately perpendicular. This contrasts with the (R)- and (S)-emixustat-bound structures, in which the cyclohexyl ring was rotated approximately 55° clockwise when viewed along the cyclohexyl-methoxy bond. The conformation adopted in the racemic structure resulted in the presence of van der Waals and hydrophobic interactions between a lipophilic pocket formed by Ile259, Phe264, and Phe279 and the cyclohexyl moiety. In the enantiopure structures, the cyclohexyl moiety was rotated away from this lipophilic pocket toward a water-filled cavity. This conformational difference was accompanied by an inward movement of Phe264 and Phe196 toward the lipophilic cyclohexyl group, such that weak van der Waals contacts were established. In this conformation, the cyclohexyl group was also stabilized by van der Waals and hydrophobic interactions with the Thr147 Cγ and Ile259 Cβ methyl groups. The conformational
variability observed for this portion of the molecule probably relates to differences in mother liquor composition as well crystal packing. Conformational flexibility of the cyclohexyl moiety suggests that suitable modifications to this region of the molecule could lead to stronger interactions with surrounding protein residues and higher-affinity binding.

**Inhibitory effects of emixustat on visual function.** Stronger inhibition of 11-cis-retinol production by emixustat as compared with Ret-NH₂ could lead to prolonged blockage of visual pigment regeneration in vivo. To test this hypothesis, we determined the rate of visual chromophore regeneration in WT mice treated with the drug and exposed to light. Because of the similar in vitro potency of the emixustat enantiomers, only the racemic mixture was investigated in vivo. Six hours after a 95% photobleaching, mice treated with 8 mg/kg emixustat recovered 35 ± 1.7 pmol 11-cis-retinal per eye, significantly less (P < 0.02) than did mice treated with the same amount of Ret-NH₂ (118.2 ± 27.7 pmol/eye) (Figure 3A). At the same time point, 11-cis-retinal levels in untreated control animals was 540 ± 45 pmol/eye. The difference between emixustat and Ret-NH₂ was significantly greater 20 hours after bleaching, when mice treated with Ret-NH₂ had recovered approximately 68% of the visual chromophore, whereas animals treated by oral gavage with emixustat achieved only an approximate 12% recovery (Figure 3A).

Additionally, the inhibitory effect of emixustat lasted longer; a single dose of 8 mg/kg caused nearly complete blockage of the visual cycle for over 7 days (Figure 3B). This effect could be attributed to a shifted equilibrium between the free and acylated form of emixustat. Unlike Ret-NH₂, which can be detected predominantly in its acylated form (39), eye extracts from mice treated with emixustat revealed substantial amounts of free emixustat in addition to the corresponding amides (Figure 3C).

**Protective effects of primary amines against light-induced retinal degeneration.** To conduct a side-by-side evaluation of the protective effect of selected primary amines in an animal model for acute light-induced retinal degeneration, emixustat, Ret-NH₂, or QEA-B-001-NH₂ was administrated to 4-week-old Abca4⁻/⁻ Mice were bleached with an appropriate amount of light and subsequently treated with the drug (30 mg/kg) immediately following the bleaching procedure. Six hours after the treatment, mice were sacrificed, and the visual pigment regeneration was assessed using a high-resolution fluorescence imaging system. The results showed that mice treated with QEA-B-001-NH₂ had a significant recovery of visual pigment regeneration compared to the control group, while emixustat and Ret-NH₂ showed moderate protective effects. These findings suggest that primary amines, such as QEA-B-001-NH₂, may be a promising target for the development of novel therapeutic agents against light-induced retinal degeneration.
Rdh8−/− mice. QEA-B-001-NH2 (Figure 1A), a primary amine incapable of inhibiting visual function (Figure 1B) and a substrate for LRAT, was used as a protective agent without strong inhibition of RPE65. Damaging retinal illumination (10,000 lux for 1 hour) was carried out 24 hours after drug administration. OCT images then were recorded after a 3-day dark adaptation period. In contrast to the 10-times higher potency of emixustat for inhibition of RPE65 in vitro, a lower dose of emixustat (2 mg/kg) did not completely protect the outer nuclear layer (ONL) in Abca4−/− Rdh8−/− mice, whereas 8 mg/kg of either emixustat or Ret-NH2 maintained both ONL thickness and light-scattering properties (Figure 4A). The average ONL thickness in mice receiving the 8 mg/kg dose was about 29 ± 12 μm for emixustat and 29 ± 21 μm for Ret-NH2 (Figure 4B), about 22 ± 18 μm (40) smaller than the ONL thickness of healthy mouse retina, indicating at least partial protection of photoreceptors. Abca4−/− mice, Ret-NH2, and QEA-B-001-NH2, we pretreated these double-KO mice with these compounds before exposing them to bright light. On day 2 after such exposure, TPM was used to document the formation of enlarged photoreceptor outer segments in the retina (Figure 5). On average, we counted 43,242 enlarged photoreceptors per mm² in the eyes of mice exposed to light and treated with vehicle only (soybean oil). Here, there were 438 enlarged photoreceptors per mm² in mice treated with emixustat; 2,914/mm² in Ret-NH2–treated mice; 19,109/mm² in QEA-B-001-NH2–treated mice; and none in mice unexposed to light. The increase in fluorescence of the RPE in amine-treated mice is consistent with previous reports (35). Here, we found that the RPE fluorescence in emixustat-treated mice was 73.5 ± 11 gray values, and in the Ret-NH2–treated group, it was 118 ± 6 gray values. Taken together, these results indicate that photoreceptors were the targets for the protective action of the tested compounds.

**Formation of retinylidene-emixustat Schiff base in vivo.** Formation of a covalent bonded Schiff base (imine) is reversible under physiological conditions. Such formation requires 2 spatially arranged functional groups, a nucleophilic amino group and an electrophilic carbonyl group. For our selected compounds, the efficiency of imine formation depends on numerous factors including the chemical structures of the amine and aldehyde, solvent composition, pH, and temperature. Examples of Schiff base formation in ocular tissue include transient formation of N-retinylidene-phosphatidylethanolamine, a precursor of A2E in ROS of photoreceptors. Importantly, the propensity for Schiff base formation can be used to therapeutic advantage. Drugs that contain primary amines can react with an excess of free atRAL to form a reversible Schiff base, thus lowering the effective concentration of atRAL and preventing light-induced retinal degeneration (32). To verify whether emixustat, in addition to inhibiting RPE65, can potentially act as a scavenger for free atRAL, we investigated the formation and stability of its retinylidene Schiff base. As illustrated in Figure 6, A and B, this conjugation occurred readily under a broad range of primary amine/atRAL ratios. Thus, in contrast to Ret-NH2, the reaction equilibrium strongly favors efficient Schiff base formation between emixustat and atRAL.

To evaluate whether conjugates between atRAL and emixustat are formed in vivo, we examined the retinoid composition of eyes...
from Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup> mice gavaged with 8 mg/kg of the amine 2 hours prior to light exposure (10,000 lux for 15 minutes). After organic extraction, retinoids and retinyl imines were separated by HPLC and identified on the basis of a comparison of their elution times with those of synthetic standards and their characteristic maximum UV/visible (UV/Vis) absorption at 450 nm upon protonation. Additionally, the molecular identity of retinylidene-emixustat was confirmed by tandem mass spectrometry (MS/MS) (Figure 6, C-F). These analyses clearly indicated that systemic administration of emixustat causes Schiff base formation with atRAL within mouse eye tissue. Notably, retinylidene-emixustat was detectable at very low doses, e.g., 8 mg/kg of the compound. Thus, the higher efficiency of Schiff base formation observed in vitro was confirmed by our in vivo findings. This observation indicates that emixustat can moderate atRAL concentrations upon photodeactivation of rhodopsin and thus prevent photoreceptor degeneration.

**Absence of the retinal protective effect of an emixustat derivative lacking the ability to sequester free retinal.** To further discriminate between the atRAL sequestrant and RPE65 inhibitory mechanisms of action of emixustat in retinal protection, we sought to identify a compound specifically lacking the former activity. The amine moiety of emixustat is critical for its ability to react with atRAL to form an innocuous Schiff base conjugate. On the basis of the RPE65-emixustat crystal structures, we reasoned that substitution of the primary amine with a hydroxyl moiety could maintain high-affinity RPE65 binding, while eliminating atRAL scavenging activity (Figure 7A). This diol derivative, referred to as MB-002, was active as an RPE65 inhibitor both in vitro and in vivo (Figure 7, B and C and Supplemental material), with potencies comparable to that of Ret-NH<sub>2</sub>. The incomplete in vitro inhibition was likely due to its esterification by LRAT at higher concentrations. The crystal structure of RPE65 obtained in the presence of MB-002 revealed that this compound was indeed bound to the active site in a fashion similar to that of emixustat (Figure 8). Surprisingly, the electron density in the cavity occupied by palmitate in the emixustat-bound structures indicated the presence of a second active site–bound MB-002 molecule. MB-002 bound in this secondary site forms a direct interaction with the iron cofactor, a hydrogen-bonding interaction with Tyr338, and a number of van der Waals contacts with non-polar side chains (Figure 8). The electron density was less well defined for the molecule at this secondary site, and binding at this position may have been enabled only by the high concentration of MB-002 (5 mM) used in the crystallization trials. Despite its strong and direct inhibition of retinoid isomerase activity, MB-002 failed to protect mice from light-induced retinal damage, as assessed by OCT and TPM (Figure 7, D and E). Moreover, to confirm that differences in the protective effects of emixustat versus MB-002 against light-induced retinal degeneration are not limited to mice lacking ABCA4 and RDH8, we compared the impact of these compounds on retina preservation in BALB/c mice (41). Again, we found that treatment with emixustat fully protected the retina from light-induced damage, whereas the protection conferred by treatment with MB-002 was minimal (Figure 9). Even though in these mice we had to use double the intensity of light and double the duration of light exposure as compared with the exposure used in Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup> mice, there were fewer damaged photoreceptors than in Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup> mice. These experiments in mice with pharmacologically suppressed visual cycles demonstrate that light-induced retinal degeneration in mice is predominantly induced by atRAL that is released from visual pigments after the initial photobleaching rather than from visual pigments that were regenerated during the period of light exposure.

**Discussion**

The development of safe and effective small-molecule therapeutics for blinding retinal degenerative diseases still remains a major challenge (42). Several different approaches targeting specific enzymes or signaling pathways have yielded a number of promising leads, among which is modulation of visual cycle function (43, 44). Two experimentally validated methods for prevention of light-induced retinal degeneration involve (a) inhibition of the retinoid cycle (7, 30) and (b) sequestration of excess atRAL by drugs containing a primary amine group (32). Conceptually, these methods are derived from 2 different ideas but have a com-
mon goal of reducing atRAL and/or atRAL condensation product toxicity as discussed below.

Blocking of the visual cycle would slow down rhodopsin regeneration through the retinoid cycle and, in turn, lower the production of toxic atRAL and its conjugation product A2E. For this mode of action, a low concentration of a potent inhibitor would be most desirable. An example of such an RPE65 inhibitor derived from Ret-NH₂ is emixustat, which is currently undergoing clinical trials (21, 22). Comparison of these 2 primary amines indicated a 15-fold greater inhibitory potency of emixustat in our standard retinoid isomerization assay (Figure 1B). However, marked suppression of the visual cycle is predicted to have highly undesirable consequences. Prolonged inhibition of 11-cis retinoid production causes accumulation of unliganded opsin, a condition that resembles Leber congenital amaurosis (LCA) or vitamin A deprivation, and this gives rise to retinal dystrophies as demonstrated in mouse models (24, 26). In particularly, cone photoreceptor cells, key cells for supporting human vision in a modern world bathed in artificial illumination, are perilous to chromophore deprivation (26, 45–48). Thus, precise dosing is critical for drugs with potent detrimental RPE65 inhibitory properties and possessing protective sequestration activity.

The crystal structures of RPE65 in complex with emixustat provide a molecular basis for understanding its strong inhibitory activity. Emixustat interacts with the retinoid-binding site of RPE65 and thus can be structurally classified as a competitive inhibitor. The hydroxyl group of the emixustat chiral center is appropriately positioned to participate in a hydrogen-bonding interaction with Thr147, an interaction that is not possible for Ret-NH₂. The importance of this polar interaction is evident from the preferential binding of (R)-emixustat in the presence of an equimolar concentration of (S)-emixustat as well as from the more chemically favorable and better-ordered binding of (R)-emixustat compared with that of (S)-emixustat in structures obtained from enantiopure compounds. Appropriate positioning of the primary amine group in emixustat allows ion-pairing interactions with Glu148 and the bound palmitate carboxylate groups. Because Ret-NH₂ is extended by 1 methylene group compared with emixustat, it is conceivable that this ion-pairing interaction may be less favorable for Ret-NH₂. The 2 different crystal forms reported here
μM of aTRAL, a highly reactive compound that is cytotoxic to any cell it contacts (29–32, 49). Extensive studies in animals, including rats as well as WT and Abca4−/−Rdh8−/− double-KO mice that closely mimic many features of human retinal degeneration, showed that Ret-NH2 exhibits a protective effect against light-induced damage by preventing buildup of aTRAL and its condensation products (7, 33, 50, 51). But, Ret-NH2 is (a) used as a substrate by LRAT, (b) a less potent inhibitor of RPE65 than emixustat, and (c) metabolized to retinol in vivo (7, 26, 33, 35, 39, 50, 52–54). First, Ret-NH2 is retained in the eye by the action of LRAT that produces its amidated precursor, and then the resulting retinyl amide is slowly hydrolyzed to evoke a long-lasting suppression of retinoid isomerase activity (35). Emixustat demonstrate that the cyclohexyl moiety of emixustat has a significant degree of conformational flexibility within the RPE65 active site. Many of the residues in close proximity to this portion of the molecule belong to a mobile region of RPE65 that also serves to anchor the protein to microsomal membranes. Thus, it is likely that conformational differences in these regions alter the shape of the cyclohexyl-binding site, which in turn gives rise to the 2 distinct conformations observed in the crystal structures. Should it be desirable to enhance the emixustat binding affinity for RPE65, it would appear that the methylcyclohexyl moiety would be a strong candidate site for chemical modification.

The second strategy involves sequestration of aTRAL. Rod photoreceptor cells express huge amounts of rhodopsin estimated at a level of 3 to 5 mM (4). Thus, a 10% bleaching will liberate 300–500 μM of aTRAL, a highly reactive compound that is cytotoxic to any cell it contacts (29–32, 49). Extensive studies in animals, including rats as well as WT and Abca4−/−Rdh8−/− double-KO mice that closely mimic many features of human retinal degeneration, showed that Ret-NH2 exhibits a protective effect against light-induced damage by preventing buildup of aTRAL and its condensation products (7, 33, 50, 51). But, Ret-NH2 is (a) used as a substrate by LRAT, (b) a less potent inhibitor of RPE65 than emixustat, and (c) metabolized to retinol in vivo (7, 26, 33, 35, 39, 50, 52–54). First, Ret-NH2 is retained in the eye by the action of LRAT that produces its amidated precursor, and then the resulting retinyl amide is slowly hydrolyzed to evoke a long-lasting suppression of retinoid isomerase activity (35). Emixustat demonstrate that the cyclohexyl moiety of emixustat has a significant degree of conformational flexibility within the RPE65 active site. Many of the residues in close proximity to this portion of the molecule belong to a mobile region of RPE65 that also serves to anchor the protein to microsomal membranes. Thus, it is likely that conformational differences in these regions alter the shape of the cyclohexyl-binding site, which in turn gives rise to the 2 distinct conformations observed in the crystal structures. Should it be desirable to enhance the emixustat binding affinity for RPE65, it would appear that the methylcyclohexyl moiety would be a strong candidate site for chemical modification.

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Figure 6. Emixustat-aTRAL Schiff base formation in vitro and in vivo. (A) Chromatographic separation and detection of primary amines, aTRAL, and their Schiff base conjugates. Emixustat or Ret-NH2 was incubated with aTRAL for 2 hours, and the mixture was separated on a C-18 column. Representative HPLC chromatograms indicate the formation of an emixustat Schiff base (peak “a”) and Ret-NH2 Schiff base (peak “b”) at an aldehyde/amine ratio of 1:1. Peak “c” corresponds to unreacted aTRAL. (B) Quantification of Schiff bases formed upon incubation of increasing concentrations of aTRAL with either 0.2 mM emixustat (inverted triangles) or Ret-NH2 (circles). (C) The chromatogram illustrates separation of an emixustat Schiff base standard. Peak 1, between 6 and 7 minutes, corresponds to the emixustat retinylidene Schiff base as indicated by its MS spectrum (D) with a dominant ion of m/z = 530.5 corresponding to [MH]+ of the emixustat-retinylidene Schiff base. Peak 2 corresponds to aTRAL. Inset in D shows the MS/MS fragmentation pattern of the m/z = 530.5 parent ion. (E) Detection of emixustat-retinylidene Schiff base in vivo; 8 mg/kg emixustat was administered to Abca4−/−Rdh8−/− mice 2 hours before light bleaching. After bleaching, mice were euthanized, and eye extracts were analyzed by LC-MS. Peak 1′ observed in the chromatogram corresponds to an emixustat Schiff base. Its identity was confirmed by its UV/Vis spectrum with a characteristic absorbance maximum of 462 nm (inset) and by MS (F), in which fragmentation of the m/z = 530.5 ion yielded the product ion at m/z = 438.5, identical to that shown in D.
also served as a moderate LRAT substrate and thus was absorbed by RPE cells as well (Figure 1C). Interestingly, although the efficacy of emixustat against retinal degeneration in Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup> mice did not differ from that observed for Ret-NH<sub>2</sub> and directly affected photoreceptor cells (Figure 5), the inhibitory effect on the retinoid cycle persisted much longer, suppressing production of the visual chromophore. Thus, the potential risk of adverse effects on photoreceptor health caused by prolonged inadequate regeneration of the visual chromophore appears to be much greater with emixustat, at least in mice (24, 26). We have also demonstrated that emixustat forms a surprisingly stable Schiff base with atRAL in vitro and that this complex can be trapped in vivo (Figure 6). Moreover, the protective effects of emixustat against light-induced retinal damage appear to be critically dependent on its ability to sequester atRAL, as evidenced by the failure of its diol derivative, MB-002, to protect against photoretinopathy in mice (Figure 7).

The disadvantage of this sequestration approach is the required high dose of the amino group–containing compound. Thus, a lead molecule must possess a high safety profile, achieve therapeutic inhibitory concentrations in the eye through reversible conjugation with atRAL, and only partially interfere with retinoid processing. If the conjugation between the amine and retinal is too avid, this could ultimately lead to toxicity due either to the formed product or to prolonged suppression of 11-cis-retinal production. Thus, only a partial slowing, but not a complete blockade, of visual chromophore regeneration, together with retinal sequestration activity, appears to be a viable therapeutic strategy for the prevention of many retinal degenerative diseases.

**Methods**

*Synthetic chemistry.* Synthesis of enantiopure emixustat and MB-002 was carried out as described in the supplemental material (Supplemental Figures 3–5).

**TPM evaluation of the impact of retinoid cycle modulators on photoreceptor protection from light-induced retinopathy.** For these experiments, we used 4- to 5-week-old Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup> and BALB/c mice. After administering either 80 mg/kg Ret-NH<sub>2</sub>, 80 mg/kg QEA-B-001-NH<sub>2</sub>, 40 mg/kg emixustat (racemic), 80 mg/kg MB-002, or 100 μl soybean oil with less than 10% (v/v) DMSO (control group), mice were placed in a dark room for 12 to 16 hours. Next, Abca4<sup>−/−</sup> Rdh8<sup>−/−</sup>
mice were exposed to bright light at 10,000 lux for 1 hour, and BALB/c mice were exposed to 20,000 lux for 2 hours as described below. These mice and a control group treated with soybean oil but not exposed to bright light were then kept in the dark for 2 days. On day 2 after light exposure, TPM was performed on intact eyes to evaluate the impact of the treatments on photoreceptors. A Leica TCS SP5 with Vision-S (Coherent) was used as described previously (31). All images were obtained with 730 nm excitation, 5 mW laser power, and the same settings of the detector and scanning parameters. Raw TPM images were used to quantify the impact of treatment. Enlarged photoreceptors were counted in the central/superior portion of the retina, 9–12 μm beneath the RPE, in an image frame corresponding to roughly 92 × 92 μm. 3D sections were reconstructed from the series of z-stack images using Leica LAS-AF offline analysis software, version 3.1.2, for Leica TCS SP8.

RPE microsomal preparations. Bovine RPE microsomes were isolated from RPE homogenates by differential centrifugation as previously described (55). The resulting microsomal precipitate was resuspended in 10 mM Bis-Tris propane/HCl buffer, pH 7.4, to achieve a total protein concentration of approximately 5 mg/ml. Then, the mixture was placed into a quartz cuvette and irradiated for 6 minutes at 4°C with a ChromatoUVE transilluminator (model TM-15; UVP) to eliminate residual retinoids. After irradiation, DTT was added to the RPE microsomal mixture to achieve a final concentration of 5 mM.

RPE65 crystallization and structure determination. Bovine RPE microsomes were isolated as previously reported (56). Proteins were extracted from the microsomes with C₆E₆, and RPE65 was purified from the sample by ion-exchange chromatography (56). Racemic, (R)- or (S)-emixustat, or MB-002, delivered in N,N-dimethylformamide (DMF), was added to the microsomal suspensions to achieve a final concentration of 1 to 2 mM during the detergent extraction portion (DMF), was added to the microsomal suspensions to achieve a final concentration of 1 to 2 mM during the detergent extraction portion. After 4°C, the mixture was placed into a quartz cuvette and irradiated for 6 minutes at 4°C with a ChromatoUVE transilluminator (model TM-15; UVP) to eliminate residual retinoids. After irradiation, DTT was added to the RPE microsomal mixture to achieve a final concentration of 5 mM.

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Crystallization trials were performed by the hanging-drop vapor diffusion method using the Wizard Cryo 1 sparse matrix screen (Rigaku). Crystals were typically observed under several conditions. Well-diffracting, untwinned crystals were obtained under conditions no. 11 [35% v/v MPD, 200 mM (NH₄)₂SO₄ and 100 mM Tris-HCl, pH 8.5]; no. 26 (40% v/v PEG 300, 200 mM NaCl and 100 mM CHES-NaOH, pH 9.5); and no. 15 (50% PEG 400, 100 mM CHES, pH 9.5, and 200 mM NaCl). These crystals grew to a final size of approximately 50 × 50 × 200 μm within 1 week. Crystals were harvested with micro-loops (MiTeGen) and flash cooled in liquid nitrogen prior to x-ray exposure. No cryoprotective agents other than those present in the mother liquor were needed to suppress ice formation.

Diffraction data sets were collected on the NE-CAT ID-C and ID-E beamlines of the Advanced Photon Source (APS). Diffraction data were indexed, integrated, and scaled with the XDS package (57). Scaled intensities were converted to amplitudes by the French-Wilson procedure (58) as implemented in XDSCONV (57). Crystals of RPE65 obtained in the presence of racemic emixustat belonged to space group P6₁, with 1 monomer in the asymmetric unit, whereas crystals containing (R)- or (S)-emixustat or MB-002 belonged to space group P6₁, with 2 monomers in the asymmetric unit. The absence of twinning was determined with phenix.xtriage (59).

Crystals were isomorphous to previously determined RPE65 crystal structures (Protein Data Bank [PDB] accession codes 4F3A and 3FSN, respectively), which enabled structure solution by rigid body refinement. Bound emixustat and palmitate ligands were clearly visible in the initial electron density maps obtained from crystals grown in the presence of emixustat (38). Electron density maps from MB-002 crystal were consistent with 2 bound MB-002 molecules in the active site. The MB-002 molecule occupying the emixustat binding site was found in the (S) configuration, whereas electron density for the MB-002 in the palmitate binding site was consistent with the (R)-isomer. Adjustments to the protein structure and addition of water molecules were performed with Coot software (60). Restrained refinement was carried out using the REFMAC program (61). Following several cycles of manual model adjustments and computational refinement, the ligands were placed into the residual active site electron densities.
all-trans-retinol (1 μl in DMF, at a final concentration of 20 μM) was added. The resulting mixture was incubated at 37°C for 1 hour. The reaction was quenched by adding 300 μl methanol, and products were extracted with 300 μl hexanes. Production of 11-cis-retinol was quantified by normal-phase HPLC with 10% (v/v) EtOAc in hexanes as the eluent at a flow rate of 1.4 ml/minute. Retinoids were detected by monitoring absorbance at 325 nm and quantified based on a standard curve representing the relationship between the amount of synthetic 11-cis-retinol standard and the area under the corresponding chromatographic peak.

Mouse handling and drug administration. Abca4−/−Rdh8−/−double-KO mice on a C57BL/6J background were generated as previously described (28, 66). Male and female WT mice with C57BL/6J or BALB/cJ backgrounds were obtained from The Jackson Laboratory. Abca4−/−Rdh8−/−mice used in this study were homozygous for the Leu450 allele of Rpe65 as determined by a genotyping protocol published previously (67) and free of Crb1/rd8 (68) and rd/rd (69) mutations. Animals were housed in the animal facility at the Case Western Reserve University School of Medicine, where they were fed standard chow (LabDiet 5053) and maintained under a 12-hour light (~300 lux) and 12-hour dark cycle. Mice were dark

Figure 9. Impact of treatment with retinoid cycle modulators on the quantity of enlarged photoreceptors in BALB/c mice. Four- to five-week old male and female BALB/c mice were exposed to bright light at 20,000 lux for 2 hours on the day after they were gavaged with oil (control, 100 μl), emixustat (racemic, 40 mg/kg), or MB-002 (80 mg/kg). TPM images of intact BALB/c mouse eyes were obtained on day 2 after exposure to light, and representative 3D reconstructions for each group are shown in A. In each 3D reconstruction, the RPE is at the top, and the section through the photoreceptor cell layer is shown 9–12 μm below the RPE. Treatments are indicated in each image. Enlarged photoreceptor cells, indicated with red arrowheads, were noted only in animals that were exposed to light and treated with oil or MB-002. Enlarged photoreceptor cells were counted for animals in each treatment group (B). Error bars indicate SDs; n = 8, 5, 8, and 10 for the vehicle, no light−, emixustat−, and MB-002−treated mice, respectively. P values shown in the graph were calculated by ANOVA and represent the statistical significance for each treated group versus control animals exposed to light and treated by gavage with oil.
adapted overnight prior to experiments. A cohort of Rhd8−/− Abca4−/− and BALB/cj mice was used when they reached the age of 1 to 2 months, and both sexes were used evenly. All tested primary amines and visual cycle modulators were suspended in 100 μl soybean oil with less than 10% (v/v) DMSO and were administered by oral gavage with a 22-gauge feeding needle. Experimental manipulations in the dark were done under dim red light transmitted through a Kodak No. 1 Safelight Filter (transmittance >560 nm).

**Induction of acute retinal degeneration in Abca4−/− Rhd8−/− mice.** After dark adaptation for 24 hours, 4-week-old male or female Abca4−/− Rhd8−/− mice with pupils dilated by 1% tropicamide were exposed to fluorescent light (10,000 lux; 150 W spiral lamp; Commercial Electric) for 30 minutes in a white paper bucket (Papersmith) and then kept in the dark for an additional 3 days. The development of retinal degeneration was then examined by ultra-high-resolution spectral domain OCT (SD-OCT; Biopitgen) and ERG as previously described (10, 70).

**Analysis of emixustat amides in mouse eyes.** Emixustat (8 mg/kg) was administered by oral gavage to 4-week-old Abca4−/− Rhd8−/− mice, which were then kept in the dark for 24 hours. Then, mice were euthanized, and their eyes were homogenized in 1 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 50% methanol (v/v). The resulting mixture was extracted with 4 ml EtOAc. Extracts were dried in vacuo and reconstituted in 300 μl ethanol. The solution (100 μl) was analyzed by liquid chromatography–electrospray ionization–MS/MS (LC-ESI-MS/MS) (4.6 mm × 150 mm; Agilent ZORBAX Eclipse XDB-C18) with a gradient of acetonitrile in water (0%-100% in 30 minutes) as eluent at a flow rate of 0.5 ml/minute.

**Visual chromophore recovery assay.** After bright light exposure that resulted in photocactivation of 95% of the rhodopsin, mice were kept in the dark for 2 hours to 7 days. Then, the animals were euthanized, and their eyes were collected and homogenized in 1 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 50% methanol (v/v) and 40 mM hydroxylamine. The resulting mixture was extracted with 4 ml of hexanes. Extracts were dried in vacuo, reconstituted in 300 μl of hexanes, and 100 μl of the extract was injected into an HPLC system for analysis with 10% (v/v) EtOAc in hexanes as the eluent.

**MS-based detection of atRAL conjugates with primary amines.** MS was performed with an LXQ linear ion trap mass spectrometer (Thermo Scientific) coupled to an Agilent 1100 HPLC system (Agilent Technologies). Separation of retinoids was achieved on a reverse-phase C18 Phenomenex HPLC Column (250 × 4.60 mm; 5 μm) in a linear gradient of acetonitrile in water of 50% to 100% within 30 minutes at a flow rate of 1.5 ml/minute. All solvents contained 0.1% formic acid (v/v). The HPLC effluent was directed into the mass spectrometry via an atmospheric pressure chemical ionization (APCI) interface operated in the positive ionization mode. Parameters of the ionization and detection were tuned with synthetic standards to achieve the highest possible sensitivity. In eye extracts, the emixustat-retinylidene Schiff base was detected in a selected reaction monitoring (SRM) mode using m/z 530.5 → 438.5 transitions.

**Statistics.** Data representing the means ± SD for the results of at least 3 independent experiments were compared by 1-way ANOVA. Differences with P values of less than 0.05 were considered statistically significant.

**Study approval.** All animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committee and conformed to recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

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