Supplemental material

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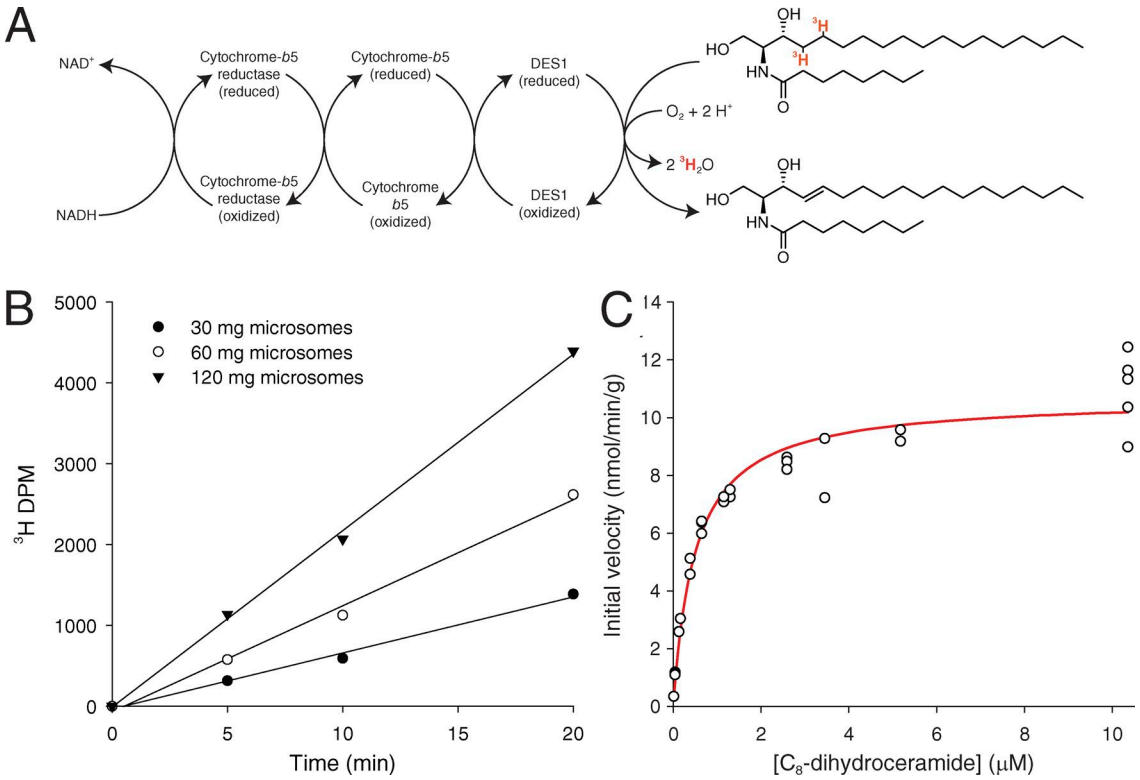

Figure S1. **Schematic and validation of the DES1 activity assay. (A)** DES1 dihydroceramide desaturase activity relies on NADH to provide electrons needed for production of an electrophilic, activated oxygen species that abstracts hydrogen from the substrate. The assay system uses a ^{3}H-4,5–labeled synthetic dihydroceramide as a substrate. DES1 activity specifically releases tritium from the substrate as water, which is separated from unreacted substrate and measured to quantify enzymatic activity. **(B)** Assessment of the linear range of the DES1 activity assay. Reactions were performed at 37°C for 0, 5, 10, and 20 min in the presence of 30, 60, and 120 mg *Lrat*$^{-/-}$ mouse liver microsomes at a fixed substrate concentration of 0.5 μM. Under these conditions, the assay was relatively linear with respect to both time and enzyme concentration. **(C)** Steady-state kinetics of mouse liver DES1 activity toward C_8-dihydroceramide. Assays were performed as described in the Materials and methods section with increasing concentrations of unlabeled C_8-dihydroceramide and a fixed quantity of 4,5-^{3}H-C_8-dihydroceramide. V_{max} and K_m values of 10.7 ± 0.3 nmol/min/g and 0.50 ± 0.07 μM, respectively, were determined for this assay system based on a total number of 28 data points measured in three separate experiments spanning a range of C_8-dihydroceramide concentrations from 0.011 to 10.4 μM.

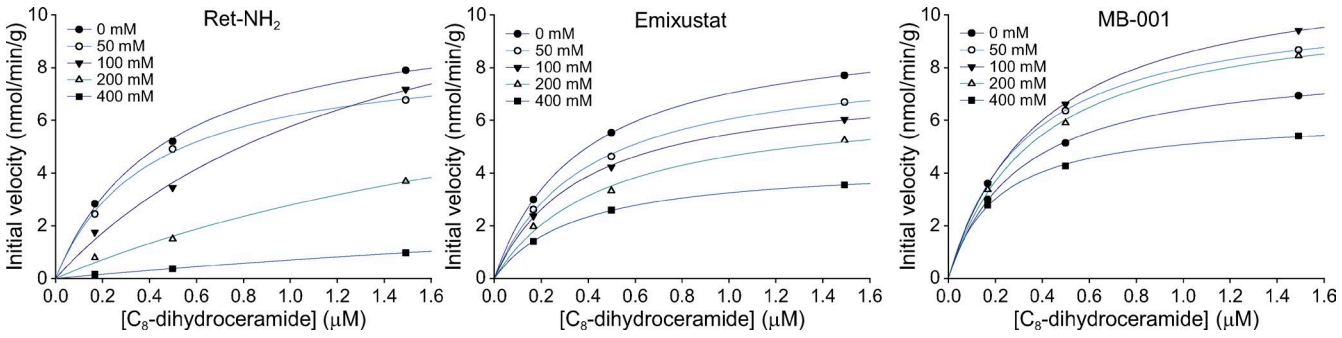

Figure S2. **Effects of RPE65 inhibitors on DES1 enzymatic function.** DES1 dihydroceramide desaturase assays were performed according to the standard method in the presence of the indicated compounds at substrate concentrations below, at, and above the K_m value for C_8-dihydroceramide. The steady-state kinetic constants and the best fit mode of reversible inhibition derived from global curve fitting along with the estimated K_i values are shown in Table S1.

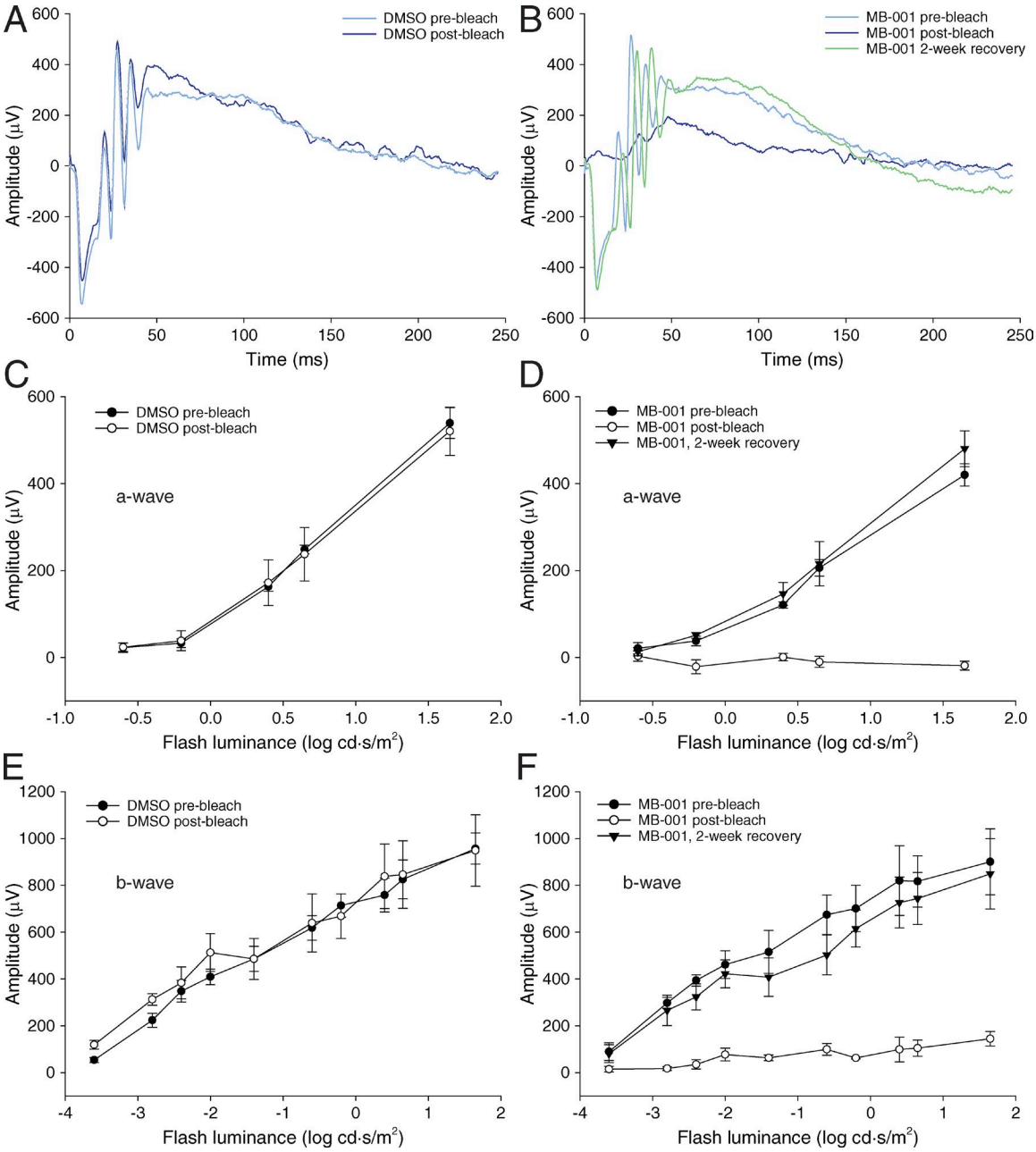

Figure S3. **Effects of MB-001 on dark-adapted mouse ERG responses. (A and B)** ERG responses to a 1.65 log cd·s/m^2 strobe flash recorded from mice (C57BL/6J, 2 mo old) that received IP DMSO vehicle or 8 mg/kg MB-001 immediately before, 22 h after, and 2 wk after a strong photobleach (10 min × 10,000 lux). **(C and D)** Plots of the scotopic a-wave amplitudes for the two groups of mice. **(E and F)** Scotopic b-wave amplitudes for the two groups of mice. DMSO group, n = 4; MB-001 group, n = 3. The data are shown as means ± SEM.

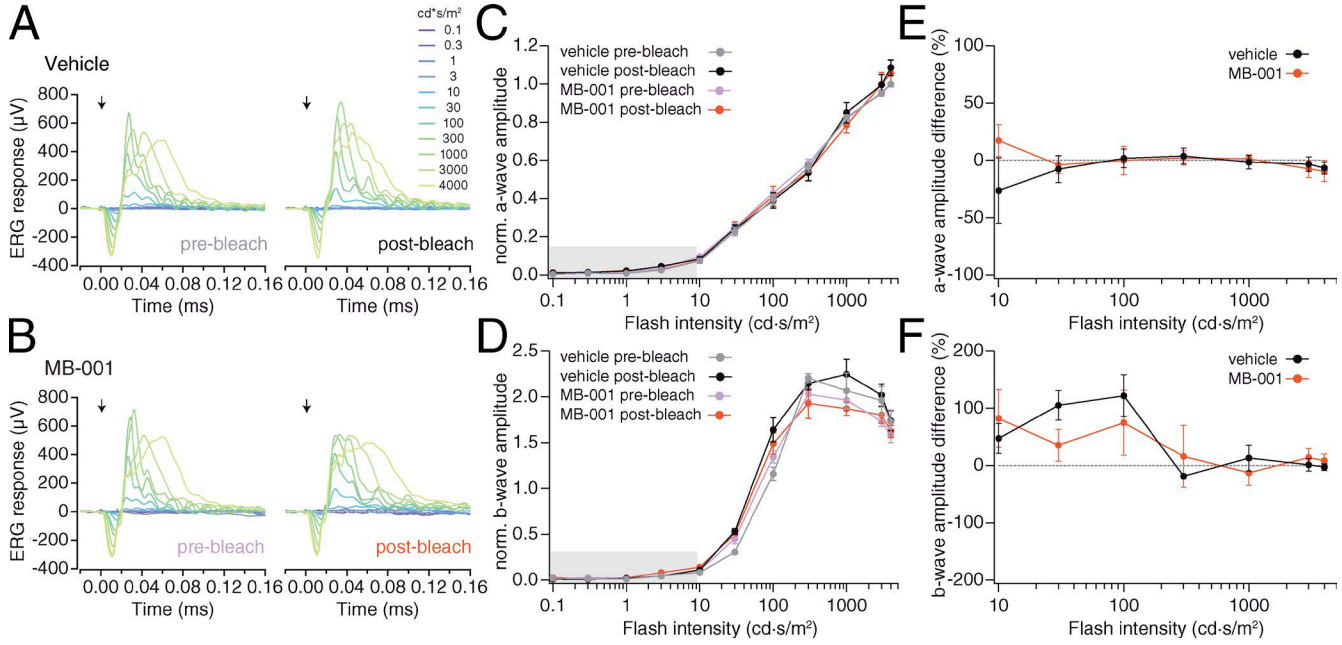

Figure S4. **Impact of MB-001 treatment on ERG responses in ground squirrels after the multiday dosing schedule used in wild-type mice. (A and B)** ERG responses from a single representative ground squirrel after vehicle (A) or MB-001 (B) treatment showing minimal effects of MB-001 treatment on cone function. ERG recordings were made in light-adapted 6-mo-old squirrels after 3 d of vehicle or MB-001 treatment before (left) and 10 min after (right) bleaching (see Materials and methods) using a brief flash of variable intensity as stimulation (black arrows, three to five trials per flash intensity). During the 3 d of treatment, animals were housed in normal cyclical lighting conditions. **(C and D)** Treatment group averages of ERG responses, which do not reveal systematic differences between vehicle- and MB-001–treated animals. We obtained mean a-wave and b-wave amplitudes at each light intensity after normalization by the a-wave amplitude to the 4,000 cd·s/m^2 flash before bleaching. We excluded responses to flash intensities <10 cd·s/m^2 (shaded regions) because they were small and corrupted by noise. **(E and F)** Changes in ERG responses induced by bleaching are not significantly different between treatment groups using this original treatment schedule. We obtained mean a-wave and b-wave amplitude differences (prebleach response − postbleach response; n = 4 squirrels; error bars represent SEM) that show no significant differences at any light intensity between treatments. We compared these ratios as paired measurements (vehicle vs. MB-001) with Wilcoxon rank–sum tests using 5% as the significance level.

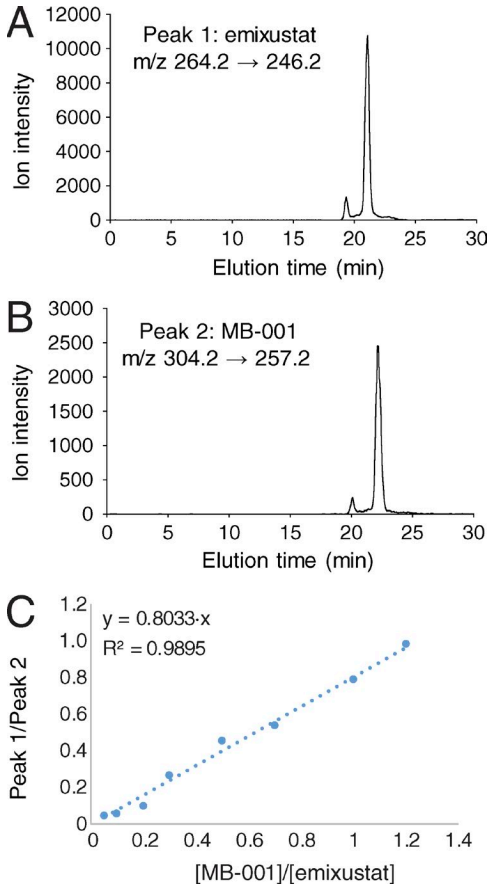

Figure S5. **Standard curve for MB-001 quantification.** 2 nmol emixustat was spiked into a serial solution of MB-001 (0–2.4 nmol) in ethanol. 100 µl of the resulting solutions was injected into a liquid chromatography/mass spectrometry system. **(A and B)** The peak areas of emixustat chromatograms, recorded by selective reaction monitoring at m/z 264.2→246.2 (A) and MB-001 at m/z 304.2→257.2 (B), were recorded. **(C)** A standard curve depicting the ratio of the corresponding peak areas of MB-001 to emixustat and the ratio of their known amounts used in the experiment.

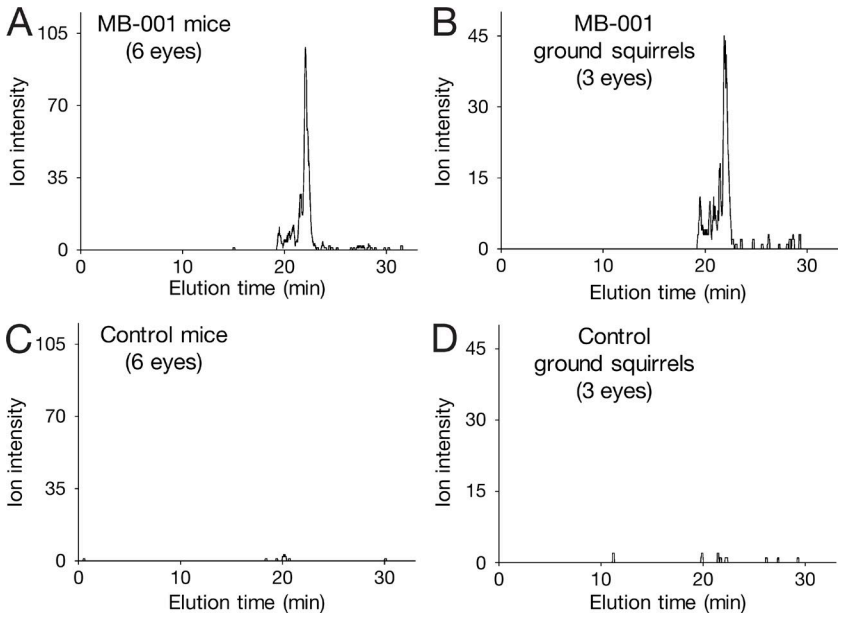

Figure S6. **Quantification of MB-001 levels in mouse and squirrel eyes.** Eye extracts were dissolved in ethanol containing 2 nmol emixustat. 100 µl of the solution was injected into the liquid chromatography/mass spectrometry system. **(A–D)** The chromatogram obtained by selective reaction monitoring at m/z 304.2→257.2 demonstrated that the eyes of C57BL/6J mice (A) and ground squirrels (B) treated with MB-001 both contained substantial amounts of the compound, although the peaks of MB-001 in the eyes from the animals treated with DMSO were negligible (C and D). The amounts of MB-001 in both animals were calculated based on the standard curve shown in Fig. S5.

Table S1. **Steady-state kinetic constants obtained for DES1-mediated dihydroceramide desaturase activity in the presence of RPE65 inhibitors**

Concentration	**Emixustat**		**MB-001**		**Ret-NH$_2$**	
µM	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
400	4.4	0.35	6.1	0.21	4.9	6.1
200	6.8	0.47	10.5	0.37	10.4	2.7
100	7.5	0.37	11.9	0.39	13.9	1.4
50	8.4	0.38	10.6	0.33	8.6	0.40
0	9.6	0.37	8.3	0.30	10.3	0.47
Mode of inhibition	Noncompetitive; K_i = 360 µM		Noninhibitory		Partial mixed or uncompetitive; K_i = ~80 µM	

V_{max} and K_m values were derived by curve fitting as shown in Fig. S2. The bottom row shows the best fit mode of inhibition and associated K_i values derived from global analysis of the data. The activity simulation and minor inhibition of DES1 activity at higher concentrations precluded global fit analysis for MB-001.