

Reductions in Serum Vitamin A Arrest Accumulation of Toxic Retinal Fluorophores: A Potential Therapy for Treatment of Lipofuscin-Based Retinal Diseases

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PURPOSE. Excessive accumulation of lipofuscin is observed in numerous degenerative retinal diseases. A toxic vitamin A-based fluorophore (A2E) present within lipofuscin has been implicated in the death of RPE and photoreceptor cells. Here, we used an animal model that manifests accelerated lipofuscin accumulation (*ABCA4*^{-/-} mutant) to evaluate the efficacy of a therapeutic approach based on reduction of serum retinol.

METHODS. *N*-(4-hydroxyphenyl)retinamide (HPR) potently and reversibly reduces serum retinol. The interaction of HPR with retinol binding protein (RBP) and transthyretin was studied by spectrofluorometry and size-exclusion chromatography. To assess the effects of HPR on visual cycle retinoids and A2E biosynthesis, HPR was chronically administered to *ABCA4*^{-/-} mice. Mice were evaluated using biochemical, electrophysiological, and morphologic techniques.

RESULTS. Administration of HPR to *ABCA4*^{-/-} mice caused immediate, dose-dependent reductions in serum retinol and RBP. Chronic administration produced commensurate reductions in visual cycle retinoids and arrested accumulation of A2E and lipofuscin autofluorescence in the RPE. Physiologically, HPR treatment caused modest delays in dark adaptation. Chromophore regeneration kinetics, light sensitivity of photoreceptors, and phototransduction processes were normal. Histologic examinations showed no alteration of retinal cytostructure or morphology.

CONCLUSIONS. These findings demonstrate the vitamin A-dependent nature of A2E biosynthesis and validate a novel therapeutic approach with potential to halt the accumulation of lipofuscin fluorophores in the eye. (*Invest Ophthalmol Vis Sci*. 2005;46:4393–4401) DOI:10.1167/iovs.05-0820

Mutations in the *ABCR* (*ABCA4*) gene are responsible for several inherited retinal and macular degenerations, including recessive Stargardt disease (STGD1) and subsets of

cone-rod dystrophy (CRD), retinitis pigmentosa (RP), and age-related macular degeneration (AMD).¹ These diseases share the phenotype of lipofuscin pigment accumulation in cells of the retinal pigment epithelium (RPE).^{2,3} The RPE plays a critical role in the support of photoreceptor cells,⁴ which includes synthesis of visual chromophore and phagocytosis of diurnally shed photoreceptor outer segments.^{5,6} Lipofuscin arises in the RPE from incomplete digestion of these retinaldehyde-rich outer-segment fragments. Lipofuscin pigments are autofluorescent because of their high retinoid content. The major fluorophore of lipofuscin is the *bis*-retinoid, *N*-retinylidene-*N*-retinylethanolamine (A2E).⁷ Dramatic accumulation of A2E has been observed in the RPE of *ABCA4* knockout mice, which represent an animal model for *ABCA4*-mediated macular and retinal degenerations.^{8–10} Still higher rates of A2E accumulation have been reported in transgenic mice with a mutation in the *elov14* gene, an animal model for dominant Stargardt disease,¹¹ and in knockout mice with mutations in the *ccl2* and *ccr2* genes, as animal models for AMD.¹² Further, postmortem specimens of RPE from patients with STGD1 contained greatly elevated A2E compared to age-matched controls.⁸

Retinal fluorophores such as A2E can be visualized in patients as fundus autofluorescence (FAF) using confocal scanning laser ophthalmoscopy (cSLO). FAF analyses in STGD1 and AMD patients have shown prominent autofluorescence and retinal dysfunction in regions immediately surrounding atrophic areas.^{13–17} Interestingly, new atrophic areas emerge within regions of intense autofluorescence, demonstrating that FAF precedes the onset of geographic atrophy.^{13,15,17} Measuring FAF by cSLO is now accepted as a diagnostic tool to monitor disease progression in STGD and AMD patients.

The biological properties of A2E have been extensively studied. Notably, A2E has been shown to possess several modes of cytotoxicity to RPE cells. For example, A2E inhibits lysosomal degradative functions in RPE phagosomes¹⁸ and predisposes RPE cells to blue light-induced apoptosis.¹⁹ At higher concentrations, A2E behaves as a cationic detergent, dissolving cellular membranes.²⁰ The first event in A2E biogenesis is condensation of all-*trans* retinaldehyde (*atRAL*) with phosphatidylethanolamine in photoreceptor outer segments. This process occurs spontaneously after light exposure. For this reason, normal mice and humans accumulate small amounts of A2E in RPE cells in an age- and light-dependent manner.^{8,10} The much faster accumulation of A2E in the above-described mouse models and humans with several forms of macular and retinal degeneration results in compromised RPE function and ultimately blindness due to photoreceptor death. Thus, the targeting of A2E accumulation in RPE cells appears a reasonable therapeutic strategy to slow the progression of visual loss in these patients.

Because A2E biosynthesis relies ultimately on circulating retinol, therapies that lower retinol should lower A2E levels. For example, leupeptin-induced lipofuscin and autofluorescence were dramatically reduced during dietary retinol deficiency.^{21–23} However, deleterious systemic effects associated

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with long-term retinol deficiency invalidate limiting dietary vitamin A as a treatment strategy. Alternatively, serum retinol can be regulated by pharmacological means. *N*-(4-hydroxyphenyl) retinamide (HPR) has been widely used as a chemotherapeutic agent for a variety of cancers, and is known reversibly to reduce serum retinol and retinol-binding protein (RBP) levels.^{24–27} Numerous clinical trials conducted over the past 20 years have shown minimal systemic effects with HPR treatment in humans.²⁸

HPR exerts its effect on retinol levels by competing for binding sites on RBP.²⁹ Dietary retinol is secreted from the liver bound to RBP. The RBP-retinol holoprotein (~21 kDa) is retained in blood by virtue of increased molecular size after binding with transthyretin (TTR, ~51 kDa).³⁰ The bulky phenyl-hydroxyl moiety of HPR (Fig. 1) may prevent the RBP-HPR complex from binding with TTR. Consequently, RBP-HPR complexes are lost to the urine through glomerular filtration. The net effect is lowered retinol and RBP in the circulation. Unlike other organs, the uptake of retinol by the eye is largely dependent on delivery by RBP.³¹ Consistently, mice with a knockout mutation in the *rbp* gene have a phenotype confined to the eyes, with no systemic signs of vitamin A deficiency.³¹

In the present study, we used *ABCA4* knockout mice to explore the potential activity of HPR to inhibit the accumulation of A2E and lipofuscin in cells of the RPE. These treated mice were evaluated biochemically, electrophysiologically, and histologically. The results suggest that HPR is effective in blocking formation of A2E and other lipofuscin fluorophores with no deleterious effects on visual function or retinal morphology.

MATERIALS AND METHODS

Materials

Holo-RBP and HPR were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-(4-methoxyphenyl)retinamide (MPR) was a gift from Robert W. Curley, Jr. (Ohio State University, Columbus, OH). 11-*cis*-

Retinaldehyde (11cRAL) was obtained from Rosalie Crouch (Storm Eye Institute, Charleston, SC) and the National Eye Institute. HPLC grade solvents were purchased from Fisher Scientific (Houston, TX). All other reagents were of highest possible purity.

Mice

Animal studies were designed to conform with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dimethyl sulfoxide (DMSO) and HPR were administered daily to wild-type and *ABCA4*^{−/−} mice by intraperitoneal (IP) injection. The phenotypic attributes of *ABCA4*^{−/−} mice have been described elsewhere.⁹ Mice were 1 to 2 months of age at study onset and were either pigmented (129/SV) or albino (BALB/c) strains. Genotyping was performed to confirm that all mice carried the leucine (wild-type) *RPE65* allele. All comparisons of the effects of HPR versus DMSO within a particular dosage group were made among littermates. HPR was delivered at concentrations of 1.5 to 15.0 μg/μL in 25 μL DMSO. Littermate controls received an equivolume of DMSO. Mice were raised under a 12-hour light-dark cycle (30–50 lux) during the treatment period and were anesthetized by IP injection of ketamine (200 mg/kg) plus xylazine (10 mg/kg) before death by cervical dislocation.

Fluorescence Quenching Spectroscopy

Fluorescence spectroscopy was performed using a commercial fluorometer (Fluorolog FL-3-22; Jobin Yvon, Edison, NJ). All titration and kinetic measurements were performed in PBS (pH 7.4) at 37°C with intermittent stirring in a 1-cm cuvette. Samples were excited at 280 nm and emission data were acquired from 290 to 550 nm with excitation and emission bandpass of 2 nm. Fluorescence emission spectra for holo-RBP (0.5 μM) were monitored over a 30-minute period. Displacement of retinol from holo-RBP was examined by adding 1 μM HPR or MPR in EtOH (0.05%, v/v) to a fresh mixture of 0.5 μM holo-RBP, followed by analysis for 30 minutes. Apo-RBP was prepared from a sample of holo-RBP (Sigma) after bleaching at 330 nm for 60 minutes (10°C) to destroy the endogenous retinol. Apo-RBP was purified and concentrated from the bleached sample using size-exclusion chromatography (SEC) as described below. Titrations of apo-RBP with MPR, HPR and retinol were performed by mixing apo-RBP (0.5 μM) with the indicated ligands (0.25–4 μM) at room temperature for 60 minutes. The fluorescence emission spectra were then acquired as described above.

Size-Exclusion Chromatography

The ability of RBP-retinol, RBP-HPR, and RBP-MPR to interact with TTR was examined by SEC. Samples were analyzed by fast protein liquid chromatography (Biological Duo Flow system; BioRad, Hercules, CA) using a 300 × 7.8-mm size-exclusion column (SEC 125; BioRad). The mobile phase (PBS, pH 7.4; 2mM Na₂N₃) was delivered at 1.0 mL/min. In these experiments, apo-RBP (5 μM) and the indicated ligand (10 μM) were mixed and incubated at room temperature for 30 minutes. After the incubation period, each sample was divided into two equal aliquots. TTR was added to one aliquot (final [TTR] = 5 μM), while the other aliquot received only TTR buffer. The samples were mixed, and incubation was resumed at room temperature for 30 minutes. After incubation, equivolume portions were removed from each sample and analyzed by SEC.

Extraction and Analysis of Serum Retinoids

Whole blood was collected from tail veins of DMSO- and HPR-treated mice at the indicated times to determine levels of serum retinol. Serum was prepared from the blood samples and retinoids were extracted using methods described by Formelli et al.^{24,25} Briefly, serum was obtained from whole blood after centrifugation at 1500 g for 10 minutes. Serum proteins were precipitated with the addition of an equivolume of ice-cold acetonitrile and centrifugation (10,000g for 10 minutes). An aliquot was removed from the soluble phase and analyzed

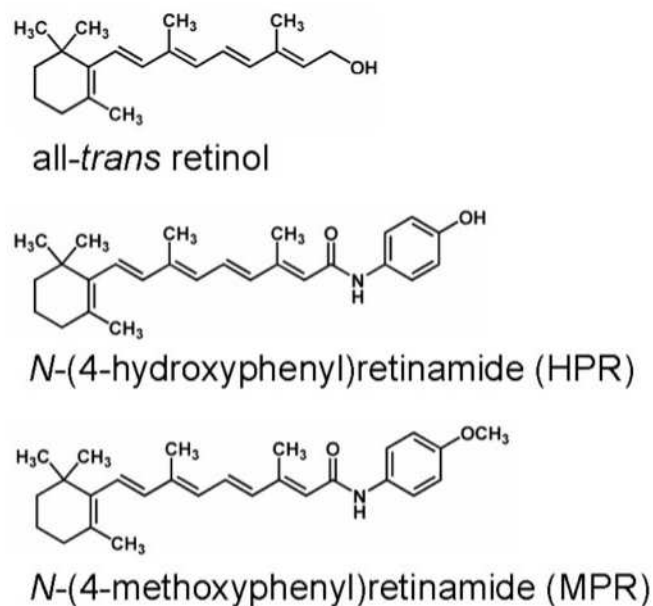


FIGURE 1. Structural similarities between all-*trans* retinol (retinol), HPR, and MPR. Each of the compounds is comprised of a β-ionone ring and a conjugated, double-bond polyene chain. HPR and MPR are distinct from retinol by virtue of their amide-linked phenyl moiety. MPR, the primary metabolite of HPR in serum, possesses a terminal methoxy group, while HPR possesses a hydroxyl group.

by HPLC using a capillary liquid chromatograph (Agilent 1100 Series; Agilent Technologies, Palo Alto, CA) equipped with a diode-array detector. Retinoids were separated on a Zorbax SB C18 5- μ m column (150 $\times$ 0.5-mm; Agilent Technologies) equilibrated with acetonitrile/water/glacial acetic acid (80:18:2, v:v) at a flow rate of 10 μ L/min.

Extraction and Analysis of Retinoids, A2E, and A2E Precursors

Steady-state levels of retinoids, A2E, and A2E precursors (A2PE and A2PE-H₂) in *ABCA4*^{-/-} mice were determined after daily administration (28 days) of either DMSO or HPR (2.5–20 mg/kg). To examine the effects of HPR on regeneration of visual chromophore, levels of 11cRAL and aRAL were measured in dark-adapted mice and during recovery from a photobleach (~1000 lux, 5 minutes), which bleached ~50% of the rhodopsin. At the indicated times, the mice were killed, the eyes were enucleated, and the posterior portion of each eye was used for extraction of retinoids or A2E and precursors. Methodologies used for extraction of A2E, A2E precursors, and retinoids from eye tissue and HPLC analysis techniques have been previously described.^{8–10} All samples were analyzed by HPLC using absorbance and fluorescence detection. In these analyses, a column thermostat was used to maintain the solvent and column temperature at 40°C. Identity of the indicated compounds was confirmed by online spectral analysis and by coelution with authentic standards.

Electroretinography

Electroretinograms (ERGs) were obtained from *ABCA4* knockout mice treated with either DMSO or HPR (10 mg/kg) for 35 days ($n = 4$ /group). ERGs were recorded using previously described methods.^{32,33} Briefly, after overnight dark adaptation, mice were anesthetized with ketamine and xylazine, their pupils were dilated with 1% atropine sulfate, and they were placed in a Ganzfeld dome (LKC Technologies, Gaithersburg, MD). ERGs were recorded from the corneal surface using a gold-loop corneal electrode and mouth-reference plus tail-ground electrodes. Responses were amplified (Grass CP511 AC amplifier; Grass Instruments, Quincy, MA) and digitized (PCI-1200; National Instruments, Austin, TX) using custom software (LabWindows/CVI; National Instruments).

Single-Flash Responses

After complete dark adaptation, rod-mediated ERGs were recorded to short-wavelength (Kodak Wratten 47A or 47B) flashes of light up to a maximum intensity of 3.32 log scot td $\cdot$ s. At the highest intensities, the leading edge of the *a*-wave of the ERG was fitted with a computational model to provide estimates of rod sensitivity *S* and *Rm_{rs}*, the maximum saturated photoresponse.^{34–36} Over a range of intermediate intensities, *b*-wave intensity-response functions were fitted with a Naka-Rushton relation to estimate *V_{max}*, the saturated *b*-wave amplitude, and *k*, the semisaturation intensity. Light-adapted cone ERGs were obtained with white flashes of light after 10 minutes of exposure to a rod-saturating background (30 cd/m²).

Recovery from Photobleach

The kinetics of recovery from a photobleach were examined by exposing the dark-adapted mice with dilated pupils to light at 500 lux for 30 seconds, which caused ~40% bleaching of rhodopsin. The time course of rod recovery was examined by monitoring the growth of the rod ERG *a*-wave to a bright probe flash (3.0 log scot td $\cdot$ s) in mice at different times after returning to darkness from 5 to 30 minutes.

Light Microscopy

ABCA4^{-/-} pigmented and albino mice were treated with either DMSO or HPR (10 mg/kg per d) for 42 days. Mice were then deeply anesthetized with 25% Avertin in PBS (pH 7.2). Whole-body perfusion was performed with a 21°C mixture of 0.1 M sodium phosphate (pH 7.4), 2% formaldehyde, and 2.5% glutaraldehyde. After 5 minutes of

perfusion, the eyes were removed, and a corneal window was cut in each eye to allow further fixation by immersion overnight at 4°C. The cornea was subsequently removed, and the hemispheres were marked for orientation. The hemispheres were fixed additionally in PBS (pH 7.4) and 1% osmium tetroxide for 1 hour, dehydrated in ethanol, and treated with propylene oxide. The hemispheres were embedded in Epon 812/Araldite 502 (2:1), and sections were cut at a thickness of 1 μ m along the vertical meridian from the superior to inferior retinal margin. The sections were stained with toluidine blue for light microscopic analysis. Images were collected with a Zeiss Axioplan microscope fitted with a Planapo 63X oil-immersion lens and a CoolSNAP digital camera.

Fluorescence Microscopy

ABCA4^{-/-} albino mice were treated with either DMSO or HPR (10 mg/kg) for 42 days. Mice were then deeply anesthetized and perfused as described above, except that the fixative was 2% formaldehyde and 1% glutaraldehyde. The eyes were removed, marked for orientation, and divided along the vertical meridian. The hemispheres were dehydrated in ethanol and embedded in LR White resin. One-micrometer-thick sections were prepared and imaged in a Zeiss Microsystems 410 confocal microscope at a laser excitation of 488 nm and emission bandwidth of 515 to 565 nm. The images were taken with a Planapo 63X oil-immersion lens and digitally processed.

RESULTS

HPR Mechanism of Action

The capacity of HPR to bind apo-RBP and subsequently affect binding to a TTR affinity resin has been reported.^{37,38} However, little information is available regarding the capacity of HPR to displace retinol from holo-RBP, the interaction of RBP-HPR with native TTR under physiological conditions, and the interaction of MPR, the principal metabolite of HPR (Fig. 1), with RBP and TTR. This latter issue is particularly important as serum concentration of MPR approaches that of HPR during chronic treatment.^{24,39} We used fluorescence-based binding assays to study RBP-ligand interaction and chromatographic techniques using native proteins to examine interaction of the RBP-ligand complexes with TTR.

We first examined the binding affinity (*K_d*) of MPR, HPR and retinol for RBP by measuring the degree of RBP fluorescence quenching during ligand titration. Both retinol and HPR demonstrated similar affinities for RBP (60 and 30 nM, respectively; Fig. 2A). MPR was also observed to bind to RBP, albeit with reduced affinity (*K_d* ~ 100 nM). We next addressed the issue of retinol displacement from holo-RBP. In these studies, HPR and MPR were added to a solution of RBP-retinol. The protein and retinoid fluorescence intensities were then monitored over time. The fluorescence spectra from a solution containing only RBP-retinol (0.5 μ M) is shown in Figure 2B. The 340-nm emission is a result of direct excitation of aromatic residues in RBP. The 470-nm emission is the result of fluorescence resonance energy transfer from RBP to retinol, which absorbs maximally at 330 nm and emits in the range of 420 to 540 nm. Figure 2C shows the effect of adding 1 μ M HPR to a solution of RBP-retinol (0.5 μ M). Like retinol, HPR absorbs maximally in the range of RBP fluorescence emission. Unlike retinol, however, HPR does not fluoresce. Therefore, the time-dependent decreases in both protein and retinol fluorescence indicate displacement of retinol by HPR. Similarly, MPR (1 μ M) also displaced retinol from RBP, but to a lesser extent than observed for HPR (Fig. 2D).

The ability of the three RBP-ligand species (RBP-retinol, RBP-HPR, and RBP-MPR) to complex with TTR was examined by SEC (Fig. 3). Unbound TTR (55 kDa) elutes at ~8 minutes and displays a single absorbance maximum at 280 nm (Fig. 3A

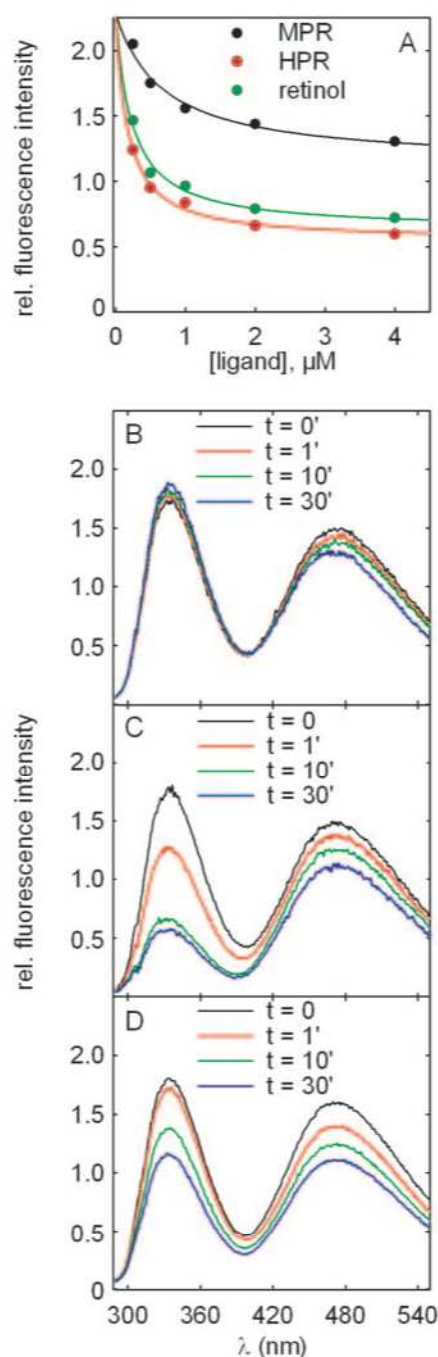


FIGURE 2. Binding of HPR, MPR, and retinol to RBP. (A) The binding affinity of HPR, MPR, and retinol for RBP (0.5 μ M) was determined by measuring the extent of RBP fluorescence quenching (280 nm excitation) in the presence of increasing concentrations of the indicated ligands. Calculation of the dissociation constants (K_d) for binding to RBP revealed similar values for retinol and HPR (~ 30 – 60 nM). The affinity of MPR for RBP was approximately twofold lower (~ 100 nM). Displacement of retinol from RBP-retinol was examined during time-based analysis of protein and retinoid fluorescence quenching. (B) Fluorescence spectra from a solution of RBP-retinol (280 nm excitation). The 340-nm emission is due to RBP, and the 470-nm emission is due to retinol. (C) Effect of adding 1.0 μ M HPR to 0.5 μ M RBP-retinol. Time-dependent decreases in both protein and retinoid fluorescence indicate displacement of retinol from RBP-retinol by HPR. (D) Like HPR, MPR can also displace retinol from holo-RBP, albeit to a lesser extent than HPR.

and inset). Under identical chromatographic conditions, RBP-retinol (21 kDa) elutes at ~ 10 minutes and demonstrates absorbance maxima at 280 and 330 nm (Fig. 3B and inset). To demonstrate interaction between TTR and RBP-retinol, representative samples of the TTR and RBP-retinol solutions (Figs. 3A and 3B, respectively) were mixed and analyzed. The resulting chromatogram showed a marked decrease in the RBP-retinol absorbance peak, an increase in the TTR absorbance peak, and an additional absorbance band (at 330 nm) in the

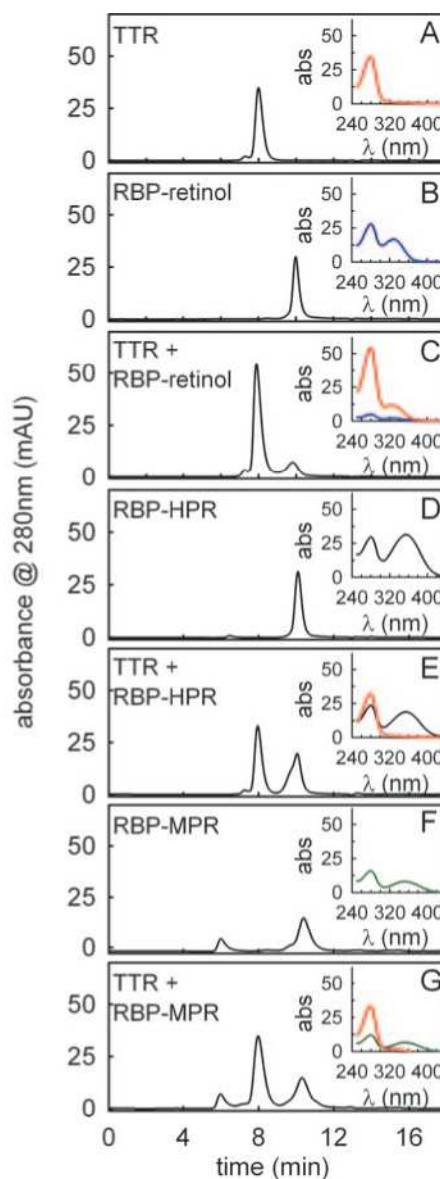


FIGURE 3. Interaction of RBP-ligand complexes with TTR. The ability of RBP-retinol, RBP-HPR, and RBP-MPR to bind with TTR was examined by size exclusion chromatography. Eluted peaks were detected by absorbance at 280 nm. Absorbance spectra (240–420 nm; insets) were obtained at the apex of each eluted peak: TTR (dark red), RBP-retinol (blue), RBP-HPR (black), and RBP-MPR (green). (A) Chromatographic and spectral properties of TTR; the indicated RBP-ligand complexes were analyzed before (B, D, F) and after (C, E, G) mixing with an equimolar amount of TTR. Interaction of RBP-retinol with TTR is readily observed as a decrease in the RBP-retinol absorbance, increase in the TTR absorbance, and the appearance of retinoid in the TTR spectra (red; C, inset). Mixtures of TTR + RBP-HPR (E) and TTR + RBP-MPR (G) showed no interaction, as evidenced by unchanged TTR absorbance and spectral properties.

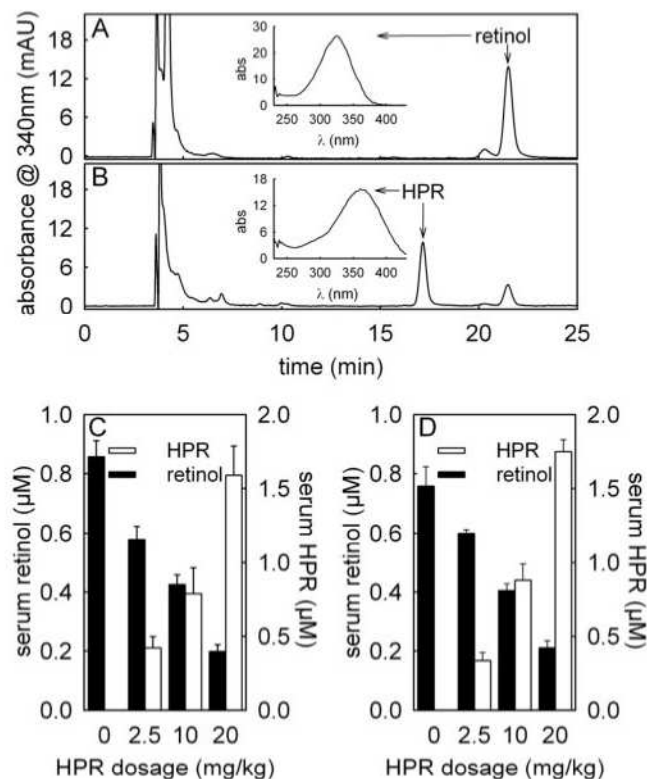


FIGURE 4. HPR-mediated reductions in serum retinol. Wild-type and *ABCA4*^{-/-} mice were administered the indicated dose of HPR in DMSO (IP) daily for 28 days ($n = 4$ mice/dosage group). Spectra show HPLC identification of retinol and HPR from sera of wild-type mice treated with either DMSO (A) or 20 mg/kg HPR (B). Histograms show an HPR dose-dependent reduction in serum retinol in wild-type (C) and *ABCA4*^{-/-} mice (D). Reductions in serum retinol correlated proportionally with reductions in RBP (not shown).

TTR absorbance spectrum (Fig. 3C and inset). These effects could only occur with the binding of RBP-retinol to TTR.

Notably, under the same experimental conditions, the RBP-HPR complex (Fig. 3D) showed no interaction with TTR (Fig. 3E). Similarly, the RBP-MPR complex did not interact with TTR (Figs. 3F and 3G). It should be noted that the lower binding affinity of MPR for RBP and its decreased water solubility (log $P = 5.672$) relative to HPR (log $P = 4.956$) contribute to aggregate formation. These MPR aggregates elute at ~6 minutes (Figs. 3F and 3G) and are well resolved from the TTR peak. Despite the relative loss of MPR due to aggregate formation, sufficient RBP-MPR was available for TTR binding; however, no binding was observed. Collectively, the data reveal that both HPR and MPR bind RBP with high affinity and generate complexes that do not associate with TTR.

HPR-Mediated Reductions in Serum Retinol

We examined the effects of HPR (2.5–20 mg/kg) on serum retinol by HPLC (Fig. 4). *ABCA4*^{-/-} mice and age- and strain-matched wild-type mice were treated with the indicated dose of HPR in DMSO daily for 28 days. Chromatographic separation and spectral identification of retinol and HPR from sera of a representative wild-type mouse treated with either DMSO (Fig. 4A) or 20 mg/kg HPR (Fig. 4B) are shown. Quantitative analysis revealed a dose-dependent reduction in serum retinol for both wild-type (Fig. 4C) and *ABCA4*^{-/-} mice (Fig. 4D). HPR doses of 2.5, 10, and 20 mg/kg reduced serum retinol by ~25%, 50%, and 75%, respectively. In all cases, reductions in serum retinol were associated with corresponding reductions in RBP as de-

termined by an RIA specific for mouse RBP (not shown). All subsequent *in vivo* analyses were performed using the intermediate HPR dose (10 mg/kg) for at least 28 days in *ABCA4*^{-/-} mice, unless otherwise indicated.

Effects of HPR on Visual Cycle Retinoids and Chromophore Regeneration

The profound HPR-induced reductions in serum retinol led us to explore the possibility that steady-state levels of visual cycle retinoids and/or visual chromophore biosynthesis might also be affected. To this end, we measured retinoid content in eyecups from light-adapted mice and chromophore regeneration in dark-adapted mice after a photobleach. The steady-state light-adapted retinoid levels in eyecup extracts are shown in Figure 5A. HPR produced a marked reduction (~50%) in each retinoid species examined and accumulated within the eye during the treatment period. It is noteworthy that the reduction of visual cycle retinoids in HPR-treated mice matched the reduction in serum retinol at this dose (Fig. 4D).

To determine whether HPR treatment caused alterations in visual chromophore (11cRAL) regeneration, we dark-adapted mice overnight and then monitored regeneration of 11cRAL and mobilization of bleached chromophore (a1RAL) after a photobleach. Steady-state dark-adapted levels of 11cRAL were ~33% lower in HPR-treated compared to DMSO-treated mice; however, there were no significant differences in regeneration kinetics after a photobleach that bleached ~50% of the dark-adapted rhodopsin (Figs. 5B and 5C).

Retinal Physiology

We examined the effects of HPR treatment on retinal structure and function by electroretinography. Parameters derived from the fit of a rod model to the leading edge of the ERG *a*-wave did not reveal differences between DMSO- and HPR-treated mice (Figs. 5D and 5E, respectively) in either the structure or function of rod photoreceptors. Correspondingly, an analysis of the rod- and cone-mediated *b*-wave intensity-response functions (Figs. 5F and 5G, respectively) also revealed no differences, indicating that retinal cells downstream of photoreceptors that contribute to the ERG were functioning normally.

The only physiological manifestation of HPR treatment was observed during recovery from a photobleach. In this series of experiments, HPR-treated mice demonstrated a delay in return of the rod photoresponse to the dark-adapted baseline levels after a 30-second photobleach (Fig. 5H). This latter result is consistent with the biochemical data showing reduced retinoid and visual chromophore levels in the HPR-treated mice.

Effects of HPR on A2E and A2E Precursor Levels

We next examined the effect of HPR on accumulation of lipofuscin fluorophores (A2E, A2PE, and A2PE-H₂) in *ABCA4*^{-/-} mouse eyecups. Analytes were separated by HPLC and measured by absorbance and fluorescence detection (Fig. 6). Representative chromatograms obtained from mice treated with either DMSO or HPR for 28 days are shown (Figs. 6A and 6B, respectively). Absorbance spectra for the indicated peaks are also provided (Fig. 6A, inset). The chromatographic tracings show that absorbance and fluorescence intensities of A2E, A2PE, and A2PE-H₂ track with each other and are dramatically reduced in the HPR-treated mouse. A2PE-H₂ is converted through an A2PE intermediate into A2E⁸ and appears before the accumulation of A2E in *ABCA4*^{-/-} mice (Mata NL et al., unpublished observation, 2005). For these reasons, we believe that A2PE-H₂ is a key A2E precursor. Accordingly, we monitored the accumulation rates of A2PE-H₂ and A2E as a function of time and HPR dose to better understand the effect of HPR on A2E biosynthesis.

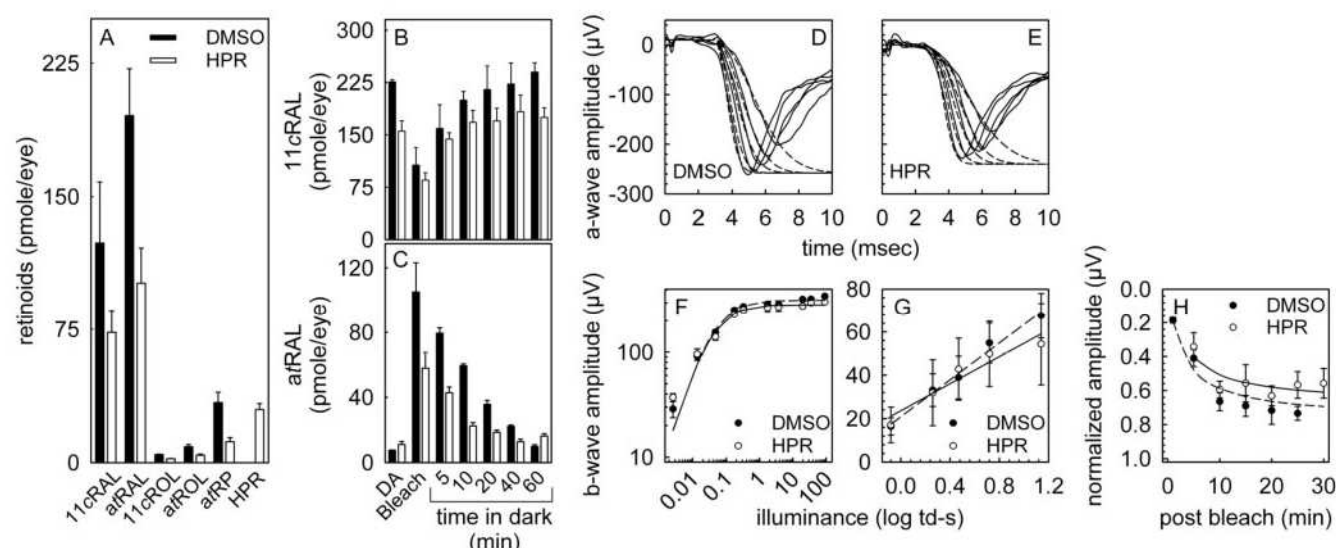


FIGURE 5. Effects of HPR on visual cycle retinoids, chromophore regeneration, and retinal physiology. Retinoid profiles were obtained for *ABCA4*^{-/-} mice treated with either DMSO or HPR (10 mg/kg) daily for 28 days. (A) Steady state, light-adapted retinoid levels. HPR produced a profound reduction (~50%) in visual cycle retinoids and accumulated within the eye during the treatment period. (B) Chromophore levels in dark-adapted mice, and (C) regeneration kinetics after a photobleach (~1000 lux, 5 minutes). HPR treatment did not significantly affect the kinetics of chromophore regeneration (B) or mobilization of the bleached chromophore (C). Electroretinography was performed on *ABCA4*^{-/-} mice treated with either DMSO or HPR (10 mg/kg) daily for 35 days. No differences were observed between DMSO- and HPR-treated mice in the parameters derived from an analysis of the rod ERG a-waves (D, E) or rod- and cone-mediated b-wave intensity-response functions (F, G). Analysis of the recovery of the rod photoresponse after photobleaching revealed a delay in return to prebleach baseline levels in HPR-treated mice (H).

We administered increasing doses of HPR in DMSO (2.5–20 mg/kg), or DMSO alone, to *ABCA4*^{-/-} mice daily for 28 days. At the indicated times, representative mice were taken from

each group for analysis. The data show significant time- and dose-dependent reductions in A2PE-H₂ (Figs. 6C, 6E, and 6G) and A2E (Figs. 6D, 6F, and 6H). Interestingly, HPR doses of 2.5,

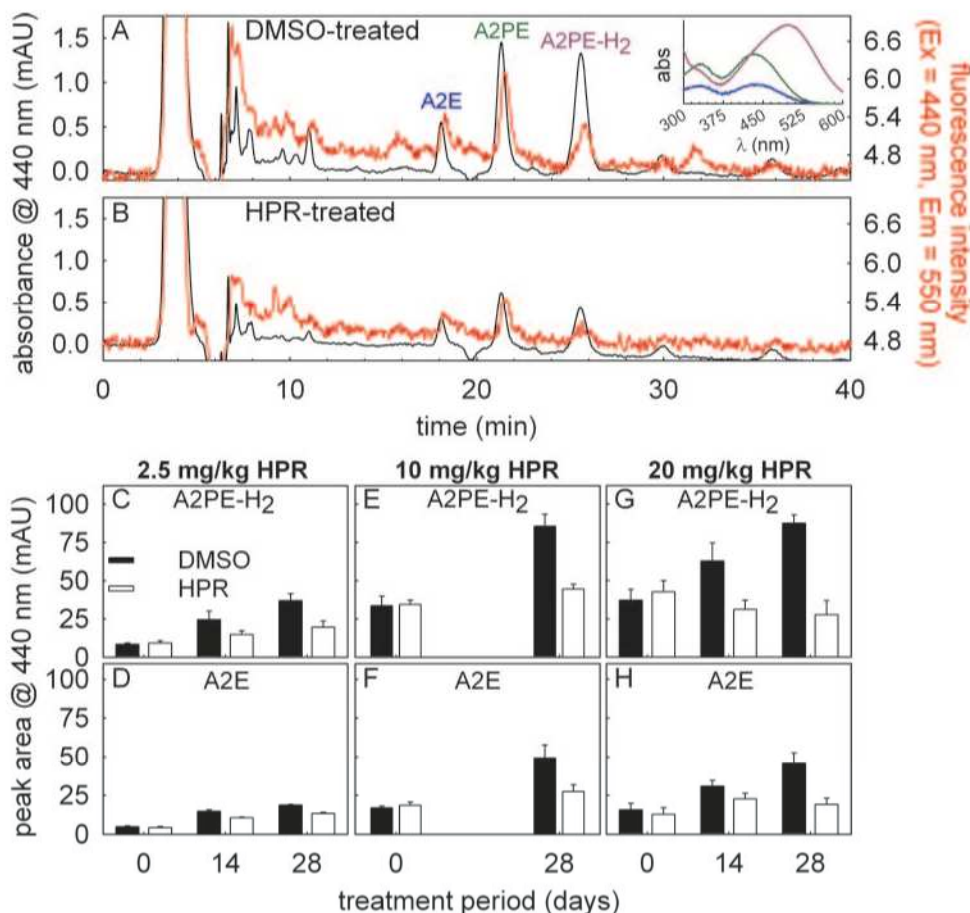


FIGURE 6. Effects of HPR on A2E and A2E precursor levels. A2E and A2E precursors (A2PE and A2PE-H₂) were extracted from eyecups of *ABCA4*^{-/-} mice and analyzed by HPLC using absorbance and fluorescence detection. Spectra are representative chromatograms from mice treated with either DMSO (A) or HPR (10 mg/kg; B) daily for 28 days. The traces show absorbance at 440 nm (black) and the fluorescence intensity obtained using 440 ± 25 nm excitation and 550 ± 25 nm emission (orange). The identity of the indicated peaks (A2E, A2PE, and A2PE-H₂) was confirmed by acquiring the absorbance spectra at the peak apex. The chromatographic data show significantly reduced A2E and precursor fluorophores in the HPR-treated mouse. An analysis of A2PE-H₂ (C, E, G) and A2E (D, F, H) accumulation as a function of HPR dose and treatment time was also performed. Mice were administered DMSO or the indicated dose of HPR daily for 28 days (*n* = 16 mice/treatment group). At study onset, mice in the 2.5 mg/kg group were aged 1 month, mice in the other treatment groups were aged 2 months. Eyecup extracts (2 eyes/sample) were prepared from representative mice in each group (*n* = 4 mice/time point) and analyzed by HPLC. The raw data obtained from HPLC peak areas (C–H) reveal a dose- and time-dependent reduction of A2E and A2PE-H₂ in HPR-treated mice.

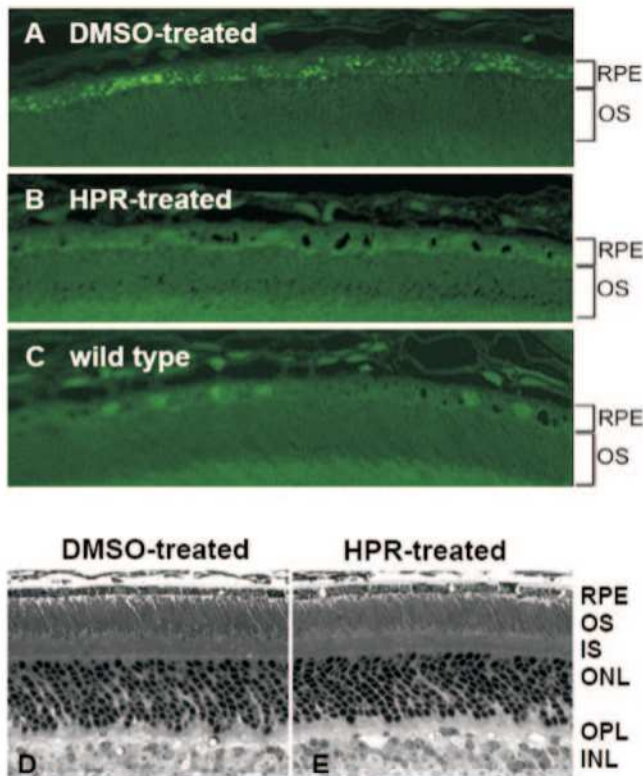


FIGURE 7. Microscopic analysis of lipofuscin autofluorescence and cytostructure of the retina. Tissue sections were prepared from the eyes of *ABCA4*^{-/-} albino and pigmented mice that had been treated with either DMSO or HPR (10 mg/kg) for 42 days. Sections from albino mice were analyzed by fluorescence microscopy, while sections from pigmented mice were used for light microscopy. Analysis of lipofuscin autofluorescence revealed considerable accumulation within the RPE of DMSO-treated mice (A). In contrast, HPR-treated mice showed significantly reduced levels of lipofuscin fluorophores (B). Tissue sections prepared from an age- and strain-matched wild-type mouse are shown for comparison (C). Analysis of RPE and retina cytostructure by light microscopy revealed no aberrant morphology associated with either DMSO (D) or HPR treatment (E). INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment.

10, and 20 mg/kg, which reduced serum retinol by ~25%, 50% and 75%, produced reductions in A2E of 29%, 45% and 60%. Thus, the percent reduction in serum retinol at each dose was comparable to the percent reduction in A2E obtained at that dose. The same trend was observed for reduction of A2PE-H₂. It is apparent from these data that a direct effect of HPR on RBP, rather than inhibition of the visual cycle, is the principal mechanism of HPR action. These findings support therapeutic efficacy for HPR to reduce accumulation of lipofuscin fluorophores and illustrate the dependence of A2E biosynthesis on serum retinol levels.

Microscopic Analysis of Lipofuscin Autofluorescence and Retinal Cytostructure

In a final study, we used fluorescence and light microscopy to examine the eyes of *ABCA4*^{-/-} mice treated with either DMSO or HPR for 42 days. The impetus for this study was to confirm data obtained from biochemical analyses of lipofuscin fluorophores and to examine the integrity of the retina and RPE after chronic HPR treatment. Analysis by fluorescence microscopy showed significant fluorophore accumulation within the RPE layer of DMSO-treated mice (Fig. 7A). In contrast, mice treated with HPR demonstrated signifi-

cantly reduced fluorophore levels (Fig. 7B). A tissue section prepared from an untreated age- and strain-matched wild-type mouse is provided for comparison (Fig. 7C). These data are entirely consistent with the biochemical data, which show profound reductions of A2E-based fluorophores in HPR-treated *ABCA4*^{-/-} mice.

Analysis of the retina and RPE cytostructure by light microscopy revealed no aberrant morphology associated with either DMSO or HPR treatment (Figs. 7D and 7E, respectively). Thus, despite the significant reduction in visual cycle retinoids, and the presence of HPR in the RPE, there is no evidence of retinotoxicity as a result of chronic HPR treatment.

DISCUSSION

HPR Mechanism of Action and Therapeutic Approach

We previously established that direct inhibition with isotretinoin (*Accutane*) of 11-*cis*-retinol dehydrogenase, which catalyzes the final enzymatic step in the visual cycle, reduces A2E and lipofuscin accumulation in the *ABCA4*^{-/-} mouse.^{40,41} Isotretinoin acts here to slow the synthesis of visual chromophore and thus to lower levels of *atRAL*, which is the primary reactant in A2E biosynthesis. However, since isotretinoin acts as a competitive inhibitor, high intracellular drug concentrations are required to achieve efficacy. The doses used in our previous study on mice (20–40 mg/kg per d)^{40,41} were far higher than doses used for treating acne in humans (0.5–2.0 mg/kg per d). Treatment of macular degeneration patients with isotretinoin at the high doses used in mice would result in unacceptable systemic toxicity. Nevertheless, these studies were useful in establishing a therapeutic approach based on modulation of intracellular retinoid concentrations.

Our investigation of alternative therapies led to HPR, a retinoic acid analog, which has been widely used over the past 20 years as a chemopreventive agent in numerous phase II and phase III cancer trials. These trials were multicenter investigations enrolling thousands of patients (aged 35–70 years) for periods up to 5 years.^{24–27} HPR was administered in doses of 200 to 800 mg/d (~2.5–10 mg/kg per d) and was deemed to be safe and well tolerated. Clinically, investigators noted reductions in serum retinol, RBP, and delayed dark adaptation.²⁷ Subsequent investigations showed a high correlation between HPR-induced reductions in serum RBP-retinol levels and manifestation of delayed dark adaptation.^{42,43}

In the present study, we performed a comprehensive analysis of the HPR mechanism of action and its capacity to reduce the accumulation of A2E-based lipofuscin fluorophores. Our investigation showed that HPR, and its primary metabolite, MPR, bind apo-RBP in a concentration-dependent manner and efficiently displace retinol from native holo-RBP under physiological conditions. Our data further showed that, unlike RBP-retinol, RBP-HPR and RBP-MPR do not associate with native TTR. These effects explain the reduction in retinol and RBP observed in clinical trials. Notably, we also found that HPR-mediated reductions in serum retinol led to proportionate reductions in toxic A2E-fluorophores. HPR doses as low as 2.5 mg/kg produced significant reductions in A2E (~30%) and precursor compounds (~50%). Finally, electrophysiologic and histologic analyses showed no deleterious functional or morphologic effects in the retina of chronic HPR treatment.

Relation between Lipofuscin Autofluorescence and Retinal Disease

Excessive accumulation of lipofuscin fluorophores in the RPE is observed in several degenerative retinal diseases (e.g., STGD1, CRD, RP, and AMD). Although the biochemical etiologies underlying these diseases are diverse, a growing body of

clinical evidence suggests that lipofuscin fluorophores contribute directly to the pathogenesis of these diseases. For example, in patients with early AMD, increased FAF is associated with reduced light sensitivity before significant retinal degeneration.⁴⁴ These fluorescent changes likely represent an early manifestation of the disease process. In addition, recent studies have established that FAF precedes the death of RPE and photoreceptor cells in STGD1 and AMD.^{13–17}

Relationship between A2E and Lipofuscin Autofluorescence

Is the FAF observed in STGD and AMD patients due to A2E and related *bis*-retinoid compounds? A2E accumulates in the RPE during normal aging,⁷ in STGD1 patients,⁸ and in several animal models of macular degeneration.^{9–12} Analysis of postmortem STGD and AMD eyes for A2E and related molecules has never been reported. However, striking similarities have been observed in the fluorescence spectra of ocular tissues from STGD and AMD patients compared to spectra from cellular structures that are known to contain A2E. For example: A2E and A2E-like compounds are the dominant fluorophores in human lipofuscin granules;¹⁶ RPE tissue from AMD-affected eyes contains fluorophores very similar to fluorophores present in lipofuscin granules⁴⁵ and in the RPE of *ABCA4*-null mice (Mata NL, unpublished observation, 2005); and the spectral properties of the long wavelength-emitting fluorophore in human fundi are similar to the spectral properties of the A2E precursor, A2PE-H₂.^{46,47}

It is well established that the genesis of ocular lipofuscin fluorophores is dependent on the presence of retinol and/or retinaldehyde (e.g., *atRAL*).^{8,10,21–23} In a recent study, a protease inhibitor was used to induce lipofuscin accumulation in the RPE of *Rpe65*-null mutant mice, which cannot generate 11c- or *atRAL*. While lipofuscin debris was observed to accumulate, there were no fluorescent species present within the debris.⁴⁸ Thus, retinaldehyde per se appears to be the requisite substrate for the formation of all lipofuscin fluorophores. It follows that therapies directed at reducing retinaldehyde content are likely to have a beneficial effect on all diseases characterized by accumulation of lipofuscin fluorophores. The fact that the percent reduction of retinaldehyde in the eyes of HPR-treated *ABCA4*^{−/−} mice (51%) was nearly identical with the percent reduction of ocular fluorophores (~50%) further supports this contention.

A final consideration is the potential for HPR to induce or inhibit mixed function oxidase systems (e.g., cytochromes) during chronic treatment. Involvement of cytochrome P₄₅₀, for example, would likely alter the pharmacokinetics of HPR and its metabolites (e.g., MPR) and therefore affect the therapeutic response. It has been shown that a 3-day pretreatment of mice with HPR (10 mg/kg) has no effect on the disposition or metabolism of HPR administered in subsequent doses or on hepatic levels of cytochrome P₄₅₀ or b₅.⁴⁹ Thus, cytochrome induction is not likely to be an issue for the direct inverse relation between HPR and serum retinol observed in the present study. Moreover, pharmacokinetic data obtained from human subjects participating in a 5-year study of HPR efficacy for the treatment of breast cancer have revealed constant drug plasma levels and constant retinol level reduction throughout the treatment period.^{24,25} In these studies, HPR and MPR levels demonstrated a direct dose-response relationship with HPR intake, and the *t*_{1/2}s of HPR and MPR did not change significantly over the treatment period.²⁵ While these data are promising for long-term treatment of lipofuscin-based retinal disease in patients in the 30- to 60-year age group, a reevaluation of HPR pharmacokinetics will be necessary for future clinical trials involving a predominantly elderly population.

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