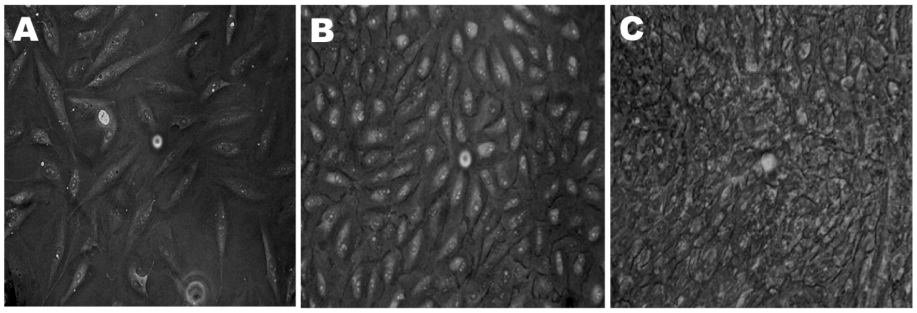
Vitamin A dimers trigger the protracted death of retinal pigment epithelium cells

Doina M. Mihai and Ilyas Washington*

Columbia University Medical Center, Ophthalmology, New York, NY 10032, USA.

Supplementary Information

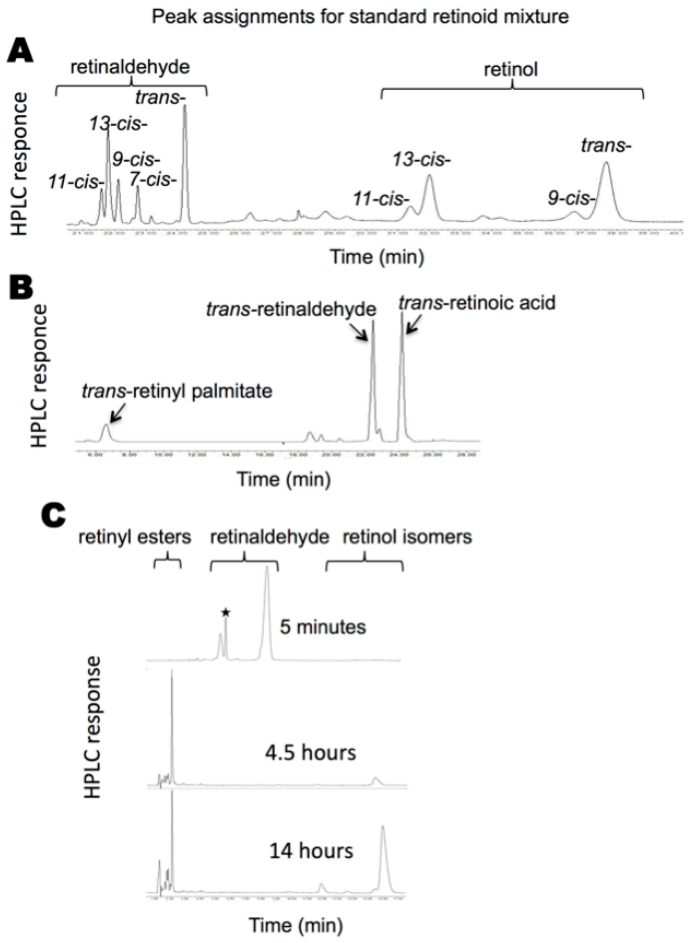


SI Fig. 1. Representative images of retinal pigment epithelium cells used **A**) Non-confluent ARPE-19 cells were grown on polystyrene. Cells were elongated with a spindle shape similar to mammalian fibroblast arrangements.

B) Two-week confluent ARPE-19 cells were grown on a polystyrene plate. Cells were denser, more polygonal and of variable size, and the number of elongated cells were decreased. This group of cells more strongly adhered to the plate, as judged by increase in trypsinization times required to detach the cells (25 min as opposed to 10-15 min for the non-confluent cells).

C) Eight-week confluent cells were grown on a gelatin-coated polystyrene plate. Cells adhered even more strongly to the plate with 40 minutes of trypsinization needed for detachment. At this stage, there were areas of multilayering as observed by 40X light microcopy. Most of the cells remained discrete (not fusiform) and somewhat polygonal.

The RPE cultures used were atypical of cells *in vivo* in that there was multilayering, they were not polarized and lacked their hexagonal packing. However cells attached strongly to the plate, a generally accepted indicator of the RPE phenotype [1], and were able to perform some normal metabolic functions of *in vivo* RPE by synthesizing and esterifying retinol. However, for the purposes of comparing relative toxicities of A2E and retinaldehyde, as well as observing morphological changes, an exact replica of the *in vivo* human RPE is not necessary nor is it possible to obtain at present.

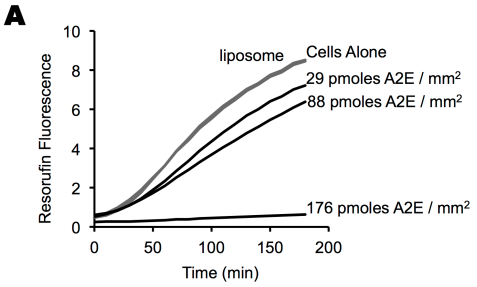


SI Fig. 2. Retinaldehyde is metabolized but A2E accumulates

A) Representative HPLC trace of a standard mixture of retinaldehyde and retinol isomers.

B) Representative HPLC trace of a standard mixture retinyl palmitate, retinaldehyde and retinoic acid. Some isomers of retinaldehyde are shown to the left of the retinaldehyde peak. Retinaldehyde and retinol isomeric mixtures were prepared according to literature procedures [2]. Briefly, we isomerized all-trans-retinaldehyde with light and reduced the resulting isomers to generate retinol isomers.

C) Representative HPLC chromatograms of retinoids isolated from the cell culture medium and cells 5 minutes, 4.5 and 14 hours after incubation with 64 pmoles of retinaldehyde/mm^2 growth area. The peak labeled with a star represents an unknown substance. Cells were grown for two weeks post-confluence on a polystyrene plate.



B

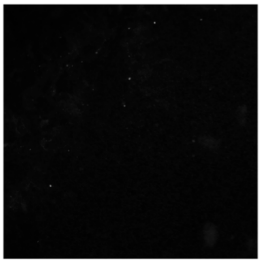
Treatment	R^2 value	EC_{50} (pmoles A2E / mm^2)	95% Confidence Interval
Sup. Fig.5B. Non-confluent with A2E	0.9526	57	50 to 61
Sup. Fig.5C. 2-week confluent with A2E	0.9953	68	58 to 79
Figure 3D. Eight-week with A2E	0.9989	66	53 to 95
Figure 3E. Non-confluent with retinaldehyde	0.9254	46	31 to 43
Figure 3F. 2-week confluent with retinaldehyde	0.9879	114	87 to 149
Figure 3G. 8-week confluent with retinaldehyde	0.9430	310	271 to 355

SI Fig. 3. Relative toxicities of A2E and retinaldehyde

A) Representative resazurin reduction curves for eight-week confluent ARPE-19 cells after being exposed to the shown amounts of A2E for one week. A2E concentrations as low as 29 pmoles/mm^2 resulted in a 13% reduction of the cell's ability to reduce resazurin compared to untreated cells. Higher concentrations of A2E (176 pmoles/mm^2) drastically reduced the rate of resazurin reduction.

B) Goodness of fit, EC_{50} and 95% confidence intervals for dose-response curves from Figure 3 of the main text.

SI Fig. 4. A2E treatment induces autofluorescence
Representative fluorescence microscopy image of control ARPE-19 cells aged for four months on polycarbonate mesh, treated weekly with liposome vehicle, fixed with methanol and imaged as in Figure 4 of the main text, using identical acquisition parameters. No autofluorescence was observed.



SI Fig. 5. A2E increases glycogen stores
Representative fluorescence microscopy image of A2E treated ARPE-19 cells fixed in methanol and reacted with pararosaniline, but not oxidized with periodic acid, and visualized using identical acquisition conditions as described for Figure 7 of the main text. No fluorescence was observed.

References

1. McKay BS, Burke JM. Separation of phenotypically distinct subpopulations of cultured human retinal pigment epithelial cells. *Experimental cell research* 1994, **213**(1)**:** 85-92.

2. Landers GM, Olson JA. Absence of isomerization of retinyl palmitate, retinol, and retinal in chlorinated and nonchlorinated solvents under gold light. *Journal - Association of Official Analytical Chemists* 1986, **69**(1)**:** 50-55.