

MOL #74732

**Dietary 9-*cis*- $\beta$ , $\beta$ -carotene fails to rescue vision in mouse models of Leber Congenital  
Amaurosis**

**Tadao Maeda, Lindsay Perusek, Jaume Amengual, Darwin Babino, Krzysztof Palczewski,  
and Johannes von Lintig**

From the Department of Pharmacology, and Ophthalmology, School of Medicine, Case Western  
Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA

**MOL #74732**

**Running Title: 9-*cis*- $\beta,\beta$ -carotene metabolism in mammals.**

**Correspondence to:** Johannes von Lintig, Ph.D., Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44160, USA. Tel: +1-216-368-3528; Fax: +1-216-368-1300. Email: Johannes.vonlintig@case.edu.

or

Krzysztof Palczewski, Ph.D., Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44160, USA. Tel: +1-216-368-4631; Fax: +1-216-368-1300. Email: kxp65@case.edu.

Text pages: 27

Number of tables: 0

Number of figures: 6

Number of references 50

Number of words in:

*Abstract* 213

*Introduction* 748

*Discussion* 1436

**Abbreviations:** 9-*cis*-R-Ac, 9-*cis*-retinyl acetate; BCMO1,  $\beta,\beta$ -carotene monooxygenase 1; BCDO2,  $\beta,\beta$ -carotene dioxygenase 2; DMSO, dimethyl sulfoxide; ERG, electroretinogram; *i.p.*, intraperitoneally; LCA, Leber congenital amaurosis; LRAT, lecithin:retinol acyl transferase; RP, retinitis pigmentosa; RPE65, retinal pigmented epithelium protein of 65 kDa; 9-*cis*-BC, 9-*cis*- $\beta,\beta$ -carotene.

## MOL #74732

### Abstract

Synthetic 9-*cis*-stereoisomers of vitamin A (all-*trans*-retinol) are especially promising agents for the fight against blinding diseases. Several studies suggest that 9-*cis*- $\beta,\beta$ -carotene (9-*cis*-BC), a natural and abundant BC isomer in the diet, could be precursor of 9-*cis*-retinoids and thus could have therapeutic applications. Here we showed that 9-*cis*-BC is metabolized both *in vitro* and *in vivo* by two types of mouse carotenoid oxygenases,  $\beta,\beta$ -carotene monooxygenase 1 (BCMO1) and  $\beta,\beta$ -carotene dioxygenase 2 (BCDO2). In the symmetric oxidative cleavage reaction at C15,C15' position by BCMO1, part of the 9-*cis*-double bond was isomerized to the all-*trans*-stereoisomer, yielding all-*trans*-retinal and 9-*cis*-retinal in a molar ratio of 3 to 1. The asymmetric cleaving enzyme BCDO2 preferentially removed the 9-*cis*-ring site at the C9,C10 double bond from this substrate providing an all-*trans*- $\beta$ -10'-apocarotenal product that can be further metabolized to all-*trans*-retinal by BCMO1. Studies in knockout mouse models confirmed that each carotenoid oxygenase can metabolize 9-*cis*-BC. Accordingly, treatment of mouse models of Leber congenital amaurosis (LCA) with 9-*cis*-BC and 9-*cis*-retinyl-acetate (9-*cis*-R-Ac), a well-established 9-*cis*-retinal precursor, showed that the *cis*-carotenoid was far less effective than the *cis*-retinoid in rescuing vision. Thus our *in vitro* and *in vivo* studies revealed that 9-*cis*-BC is not a major source for mouse 9-*cis*-retinoid production but is mainly converted to all-*trans*-retinoids to support canonical vitamin A action.

## MOL #74732

### Introduction

An enzyme-based cyclic pathway for *trans*-to-*cis* isomerization of the visual pigment chromophore all-*trans*-retinal is intrinsic to mammalian retinal rod and cone vision. This pathway, called the visual or retinoid cycle, involves two cellular compartments, both rod and cone outer segments and closely associated retinal pigmented epithelium (RPE) (von Lintig et al., 2010). In addition, cones can be supported by a pathway involving Müller cells (Fleisch and Neuhauss, 2010; Wang and Kefalov, 2011). Genetic disruption of the visual cycle in mice results in rapid or slowly progressive death of rods and cones (Travis et al., 2007). For example inactivating mutations in lecithin:retinol acyltransferase (LRAT) and retinoid isomerase (also known as RPE protein of 65 kDa (RPE65) that converts all-*trans*-retinyl esters to 11-*cis*-retinol) are associated with severe forms of retinitis pigmentosa (RP) including LCA in humans (Thompson and Gal, 2003). In contrast, perhaps because of redundancy in the redox system, mutations in retinol dehydrogenase 5 (RDH5), an enzyme responsible for oxidation of 11-*cis*-retinol to 11-*cis*-retinal, cause a mild form of retinal dysfunction called *fundus albipunctatus* and mild RP with slow dark adaptation (recovery of vision after illumination) and cone and rod degeneration (den Hollander et al., 2008; Travis et al., 2007).

Substantial efforts have been undertaken to establish therapies for patients that suffer from blinding diseases affecting the retina [for review, (den Hollander et al., 2011; Palczewski, 2010)]. Among them, a pharmacological intervention with 9-*cis*-retinoids has been successfully established in animal models and is currently undergoing evaluation in patients with different retinal diseases (Palczewski, 2010). This method relies on the fact that 9-*cis*-retinal can serve as a surrogate chromophore in 11-*cis*-retinal deficient photoreceptors (Palczewski, 2006). 9-*cis*-Retinal binds to the opsin moieties of rod and cone visual pigments, thereby preserving vision

## MOL #74732

and preventing retinal degeneration in homologous animal models for LCA (Maeda et al., 2009; Van Hooser et al., 2000).

Recent intriguing results from a clinical trial indicated that supplementation with 9-*cis*-BC, readily available as a dietary supplement powder containing 40-50% 9-*cis*-BC from the alga *Dunaliella bardawil* (Ben-Amotz et al., 1988), also can improve vision in patients that suffer from *fundus albipunctatus* caused by mutations in retinal dehydrogenase 5 (RDH5) (Rotenstreich et al., 2010). It has even been further suggested that retinal dystrophies with similar mechanisms such as various types of RP would benefit from this pharmacological intervention. The authors proposed that this effect is likely mediated by 9-*cis*-BC conversion to 9-*cis*-retinal through symmetric oxidative cleavage by the  $\beta,\beta$ -carotene-15,15'-monooxygenase (BCMO1). Interestingly, *Rpe65*<sup>-/-</sup> mice also possess some residual vision due to endogenous 9-*cis*-retinal and levels of this compound also increase when mutant mice are kept in the dark (Fan et al., 2003). Thus, an enzymatic pathway for the production of this compound could exist in mammals. However, whether 9-*cis*-BC can promote 9-*cis*-retinal production is not supported by the literature and the concept still lacks rigorous testing in animal models. Alternatively, a 9-*cis*-BC/all-*trans*-BC mixture could affect visual function because of its anti-oxidant and/or other effects (Demmig-Adams and Adams, 2002; Obulesu et al., 2011). Previous studies of 9-*cis*-BC metabolism were mainly conducted in the context of 9-*cis*-retinoic acid production. The latter compound specifically activates retinoid X receptors but also can activate nuclear hormone receptors such as the retinoic acid receptors (Heyman et al., 1992). However, the same studies reported conflicting results regarding the effectiveness of 9-*cis*-BC as a precursor for 9-*cis*-retinoids [for review, (Parker, 1996)]. Thus, 9-*cis*-BC metabolism remains to be defined in detail.

In recent years, carotenoid oxygenases have been identified as one of several key participants in carotenoid metabolism [for review, (von Lintig, 2010)]. Thus the critical importance of BCMO1 for retinoid production was demonstrated in a knockout mouse model (Hessel et al., 2007). BCMO1 is expressed in various human tissues, including the small

## MOL #74732

intestine and parenchymal cells of liver (Lindqvist et al., 2005). Moreover, BCMO1 is abundant in the RPE of human eyes and cell culture studies showed that human RPE cells can convert BC to retinoids (Chichili et al., 2005; Yan et al., 2001). Additionally,  $\beta,\beta$ -carotene-9,10'-oxygenase (BCDO2), a mitochondrial dioxygenase expressed in various tissues, can convert BC and many other carotenoids by eccentric oxidative cleavage to apocarotenoid products (Kiefer et al., 2001).

By taking advantage of novel tools and reagents, including knockout mouse models for carotenoid oxygenases, we have analyzed and now report on certain biochemical features of 9-*cis*-BC metabolism. We also compared the relative efficacy of pharmacological interventions with 9-*cis*-R-Ac and 9-*cis*- $\beta,\beta$ -carotene (9-*cis*-BC). We found that dietary 9-*cis*-BC fails to rescue vision in two mouse models of LCA.

MOL #74732

## Materials and Methods

### *Materials*

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Reagents for cDNA synthesis and quantitative real-time PCR (qRT-PCR) were obtained from Applied BioSystems (Foster City, CA). 9-*cis*-Retinal and all-*trans*-retinal were provided by Toronto Research Chemicals Inc. (Toronto, Canada). Briefly, to prepare 9-*cis*-R-Ac, 100 mg of 9-*cis*-retinal was reduced with 50 mg of sodium borohydride in 0.7 mL of EtOH at 0°C for 30 min. Solid 9-*cis*-ROL and 80 mg of 4-dimethylaminopyridine were dissolved in 0.4 ml of dry CH<sub>2</sub>Cl<sub>2</sub>, and 0.1 ml of acetic acid anhydride was added. After 6 h at 10 °C, the reaction was quenched with 0.1 ml of ethanol, CH<sub>2</sub>Cl<sub>2</sub> was removed by flowing argon at 20 °C, and 9-*cis*-R-Ac was purified by organic extraction and dried under argon (Batten et al., 2005). *Dunaliella bardawil* extracts were purchased from Nikken So Honsha Corp. (Gifu, Japan). all-*trans*-10'-β-apocartenal was a gift from Dr. Hansgeorg Ernst (BASF, Ludwigshafen, Germany).

### *Preparation of all-trans BC and 9-cis-BC from Dunaliella bardawil extracts*

All experimental procedures related to extraction, retinoid derivatization, and separation of retinoids were conducted under dim red light provided by a safelight filter (transmittance >560 nm; No. 1; Eastman Kodak). Granules in one *Dunaliella bardawil* supplement (1 capsule; 9 mg carotenes) were homogenized in a Kontes glass-glass homogenizer with 2 mL of MeOH until the granules became fine and well mixed. The resulting suspension was placed in Kimax glass tube along with 2 mL of H<sub>2</sub>O and 4 mL of hexane, capped and vortexed for 1 min prior to centrifugation for 5 min at 2,000 rpm and 4°C. The upper layer was collected and dried in a Speedvac and then dissolved in 4 mL of organic solvent composed of 75:25 MeOH:methyl *tert*-butyl ether (TBME). Sample components were separated on a ProntoSIL 200-3-C30 3.0 μm

## MOL #74732

column with 75:25 MeOH:TBME at a flow rate of 1.3 mL/min. For all purifications, 100  $\mu$ L of the above solution was injected into the ProntoSIL column. The peak fraction corresponding to 9-*cis*-BC (447 nm) was collected into a 5 mL glass tube and stored at -80°C.

### *Enzyme kinetics*

Previously described plasmids used for the expression of recombinant murine BCMO1 and BCDO2 (Amengual et al., 2011b; Kiefer et al., 2001) were transfected into *E. coli* BL21. Protein expression and tests for enzymatic activity were carried out by a published protocol (Oberhauser et al., 2008). Briefly, appropriate amounts of all-*trans*-BC and 9-*cis*-BC dissolved in hexane were mixed with 25  $\mu$ L of 12% (w/v) n-octyl  $\beta$ -D-thioglucopyranoside dissolved in EtOH and dried in a Speedvac. Then, enzyme solution (100  $\mu$ L) was added and the solution was vortexed for 20 s. Enzymatic assays were incubated at 28°C for indicated time periods and then stopped by the addition of 100  $\mu$ L NH<sub>2</sub>OH and 200  $\mu$ L MeOH. Lipophilic compounds were extracted with 400  $\mu$ L acetone and 500  $\mu$ L hexane. Extraction with hexane was repeated twice, the resulting organic phases were combined, dried in a Speedvac (Eppendorf, Hamburg, Germany) and redissolved in 200  $\mu$ L HPLC solvent. HPLC analysis was performed on normal phase ZORBAX Sil (5  $\mu$ m, 4.6x150 mm) column (Agilent) with chromatographic separation achieved by isocratic flow of 10% ethyl acetate/hexane at flow rate of 1.4 mL/min for retinal-oximes and 30% ethyl acetate/hexane at flow rate of 1.4 mL/min for  $\beta$ -10'-apocarotenal-oximes. For quantification, HPLC systems were scaled with known amounts of different all-*trans*-stereoisomer and 9-*cis*-stereoisomers of retinal-oximes or all-*trans*-10'- $\beta$ -apocarotenal-oximes, respectively.

### *Mice and 9-cis-BC, all-trans-BC and 9-cis-R-Ac administration*

Animal experiments were conducted according to accepted standards of humane care and use of laboratory animals, and were approved by the Case Western Reserve University



## MOL #74732

Animal Use and Care Committee (protocol number 2008-0074). The generation and genotype of *Bcmo1*<sup>-/-</sup>, *Bcdo2*<sup>-/-</sup>, *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice has been previously described (Amengual et al., 2011b; Batten et al., 2004; Hessel et al., 2007; Redmond et al., 1998). All knockout mouse strains and wild-type (WT) control mice had a mixed C57/BL6;129Svj genetic background. Mice at 5-6 weeks of age were injected intraperitoneally (*i.p.*) with either dimethyl sulfoxide (DMSO), 9-*cis*-R-Ac, all-*trans*-BC or 9-*cis*-BC. All injections were delivered in a total volume of 60  $\mu$ L through a 28  $\frac{1}{2}$  gauge needle. Each injection contained 0.16 mg of a test compound dissolved in DMSO and a total of 5 daily injections were given. Mice were maintained in the dark during this injection period. Animals subsequently were sacrificed under dim red safety light, and their livers and eyes were removed and immediately placed on dry ice before storage in a -80°C freezer prior to carotenoid and retinoid analyses.

### *Carotenoid and retinoid analyses*

Eyes were homogenized in 1 mL of retinoid analysis buffer (50 mM MOPS, 10 mM NH<sub>2</sub>OH, 50% EtOH in H<sub>2</sub>O, pH 7.0). Samples were allowed to incubate at room temperature for 20 min and then 1 mL of EtOH was added to stop the reaction. Five mL of hexane was added to the homogenate and vortexed for 1 min prior to centrifugation for 5 min at 2,000 rpm and 4°C. The collected hexane phase was evaporated in the SpeedVac and 300  $\mu$ L of fresh hexane was added. Samples were eluted on a normal phase HPLC using an Agilent silicon column at 1.4 mL/min using a gradient of hexanes and ethyl acetate (0-15 min: 0.5% ethyl acetate, 15-60 min: 6% ethyl acetate) as previously described (Amengual et al., 2011b). Extraction of carotenoids and retinoids as well as retinyl ester saponification of the liver were carried out as previously described (Amengual et al., 2011a).

### *RNA isolation and quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)*

## MOL #74732

RNA was isolated from mouse adipose tissue or cultured adipocytes ( $\pm$  indicated treatments) with the Trizol reagent (Invitrogen), purified by using the RNeasy system (Qiagen) and then quantified spectrophotometrically, as described previously (Lobo et al., 2010a). About 2  $\mu$ g of total RNA was reverse-transcribed by using the High Capacity RNA-to-cDNA kit and following the manufacturer's instructions (Applied BioSystems). Quantitative PCR (q-RT-PCR) was carried out with TaqMan chemistry, namely TaqMan Gene Expression Master Mix and Assays on Demand probes (ABI) for mouse *Bcdo2* (Mm00460051\_m1) and for mouse *Bcmo1* (Mm00502437\_m1), respectively. The 18S rRNA (4319413E) probe set (ABI) was used as the endogenous control. All real-time experiments were performed with the ABI Step-One Plus qRT-PCR machine. To control for between-sample variability, mRNA levels were normalized to 18S rRNA for each sample by subtracting the Ct for 18s rRNA from the Ct for the gene of interest, thereby producing a  $\Delta$ Ct value. The  $\Delta$ Ct for each treatment sample was compared to the mean  $\Delta$ Ct for control samples by using the relative quantification  $2^{-(\Delta\Delta Ct)}$  method to determine fold-changes (ABI Technical Bulletin No. 2).

### *Electroretinogram (ERG)*

All ERG procedures were performed by previously published methods (Maeda et al., 2009). Briefly, mice under a safety light were anesthetized by intraperitoneal injection of 20  $\mu$ l/g body weight of 6 mg/ml ketamine and 0.44 mg/ml xylazine diluted with 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl. Pupils were dilated with 1% tropicamide. A contact lens electrode was placed on the eye and a reference electrode and ground electrode were positioned on the ear and tail, respectively. ERGs were recorded with a computerized system, UTAS E-3000 (LKC Technologies, Inc.).

*Single-flash recording.* The duration of white light flash stimuli (from 20  $\mu$ s to 1 ms) was adjusted to provide a range of illumination intensities (from -3.7 to 1.6 log cd-s/m<sup>2</sup>). Three to five

## **MOL #74732**

recordings were made at sufficient intervals (from 3 s to 1 min) between flash stimuli to allow recovery from any photo-bleaching effects.

### *Statistical analyses*

Results are presented as means  $\pm$  SD and the number of experiments is indicated in the figure legends. For each experiment, all determinations were performed at least in triplicate. Statistical significance was assessed by using the two-tailed Student's *t*-test.

**MOL #74732**

## **Results**

### **Isolation and purification of 9-*cis*-BC**

For 9-*cis*-BC purification, we used commercially available preparations of the unicellular algae *Dunaliella bardawil* (Ben-Amotz et al., 1988). Lipids were extracted and separated by HPLC with an Agilent C30 silicon column which revealed that the extract contained two major compounds with absorption maxima and retention times described for all-*trans*-BC and 9-*cis*-BC, respectively (Supplemental Figure 1A). We then established a quantitative HPLC method for collecting each BC stereoisomer (Supplemental Figure 1B). Separated BC stereoisomers were promptly stored in DMSO at -80°C in light-tight glass vials until further use. To determine the stability of these preparations, we re-analyzed the geometric composition of isolated 9-*cis*-BC at intervals up to 10 days and found it was stable under these storage conditions (Supplemental Figure 1 B).

### **BCMO1 and 9-*cis*-BC metabolism**

To first determine whether 9-*cis*-BC can be converted by BCMO1, we first expressed recombinant murine BCMO1 in *E. coli* and then assayed this reaction by a previously established procedure (Oberhauser et al., 2008). Recombinant murine BCMO1 was incubated with 20 µM all-*trans*-BC for 5 min and lipids were isolated by HPLC. As expected, this analysis revealed that all-*trans*-BC was converted to all-*trans*-retinal (Fig. 1A,B). Aside from the all-*trans*-retinal product, trace amounts of the 13-*cis*-retinal became detectable but other retinal stereoisomers were absent. When similar assays were performed with 20 µM 9-*cis*-BC as the substrate, HPLC analysis detected that the retinal cleavage products were the all-*trans*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal stereoisomers as confirmed by co-chromatography with authentic standards (Fig. 1C,D). Moreover, production of different retinal stereoisomers from all-

## MOL #74732

*trans*-BC and 9-*cis*-BC increased with prolonged incubation (Fig. 2A,B). We next determined BCMO1 conversion rates for 9-*cis*-BC and all-*trans*-BC by incubating the same BCMO1 enzyme preparation with 20  $\mu$ M of each substrate. These analyses showed that BCMO1 metabolized all-*trans*-BC 5-fold faster than 9-*cis*-BC (Fig. 2A,B). Again, all-*trans*-BC was mainly converted to all-*trans*-retinal. With 9-*cis*-BC as substrate, all-*trans*, 9-*cis*-retinal and 13-*cis*-retinal stereoisomers were formed at a molar ratio of approx. 9:3:1 (Fig. 2B). Finally,  $K_M$  and  $V_{MAX}$  values were determined by incubating the same enzyme preparation with increasing amounts of the two BC substrates for 5 min (Fig. 2C,E). For all-*trans*-BC, the  $K_M$  value was estimated to be 53.6  $\mu$ M and  $V_{MAX}$  as 188.6 pmol retinal/min x mg. With 9-*cis*-BC, the  $K_M$  value was about 14.3  $\mu$ M and  $V_{MAX}$  was 11.8 pmol retinal/min x mg.

Thus although BCMO1 demonstrated a lower  $K_M$  value for the 9-*cis*-BC stereoisomer, it metabolized 9-*cis*-BC at a much lower rate than the all-*trans*-BC stereoisomer. Interestingly, 9-*cis*-BC was not converted to equimolar amounts of the 9-*cis*-retinal and the all-*trans*-retinal stereoisomer by cleavage at the C15, C15' position. Production of the all-*trans*-stereoisomer was 3-fold higher than that of the 9-*cis*-stereoisomer, indicating that the 9-*cis*-double bond was partially isomerized to the all-*trans* configuration during this reaction.

### BCDO2 also catalyzes the conversion of 9-*cis*-BC

Besides BCMO1, BCDO2 can contribute to 9-*cis*-BC metabolism. To analyze the putative role of BCDO2 in 9-*cis*-BC metabolism, we performed enzyme assays with recombinant murine BCDO2 by incubating the same enzyme preparation with either 20  $\mu$ M all-*trans*-BC or 9-*cis*-BC (Fig. 3A). After 5 min incubation, putative cleavage products were isolated and subjected to HPLC analysis. With all-*trans*-BC, significant amounts of all-*trans*- $\beta$ -10'-apocarotenal were produced as verified by co-chromatography with an authentic standard (Fig. 3A,C). With 9-*cis*-BC as substrate, all-*trans*- $\beta$ -10'-apocartenal was also the major cleavage product (Fig. 3A,C). Aside from this stereoisomer, another likely 9-*cis*- $\beta$ -apocarotenal cleavage product with

## MOL #74732

comparable spectrum but different retention time was produced (Fig. 3A,C). BCDO2-catalyzed turnover rates for 9-*cis*-BC and all-*trans*-BC were comparable (Fig. 3B). With 9-*cis*-BC as substrate, the all-*trans*-stereoisomer product exceeded the amount of the 9-*cis*-stereoisomer by about 7-fold, indicating that BCDO2 preferentially removed the  $\beta$ -ionone ring from the 9-*cis*-configured site of BC.

### Metabolism of 9-*cis*-BC in WT and carotenoid oxygenase-deficient mouse models

We next analyzed the contribution of each carotenoid oxygenase to 9-*cis*-BC metabolism by taking advantage of *Bcmo1*<sup>-/-</sup> and *Bcdo2*<sup>-/-</sup> mouse lines. Previously, we reported that intestinal BC absorption is repressed on vitamin A sufficient diets (Lobo et al., 2010b). Additionally, several studies indicate that intestinal absorption of 9-*cis*-BC is significantly lower than that of all-*trans*-BC. Therefore, to avoid problems with intestinal absorption, we *i.p.* injected 9-*cis*-BC dissolved in DMSO. This route of BC administration has been shown to result in efficient retinoid production in mice (Kim et al., 2011). Here we injected animals with 0.2 mg 9-*cis*-BC dissolved in 60  $\mu$ l of DMSO five times at daily intervals and maintained them continuously in the dark. After 5 days, we analyzed BC and retinoids in the liver. Whereas WT mice accumulated 9-*cis*-BC in this organ, accumulation of 9-*cis*-BC was significantly lower in *Bcmo1*- and *Bcdo2*-deficient animals (Fig. 4A). This result was surprising because previous studies indicated that BC accumulates in large quantities in *Bcmo1* knockout mice but not in WT animals (Hessel et al., 2007). A possible explanation for such accumulation was revealed when we measured mRNA expression levels of the two carotenoid oxygenases in different mouse strains. Thus in knockout mice, expression of each remaining carotenoid oxygenase were significantly increased over WT levels (Fig. 4B). Because both carotenoid oxygenases can metabolize 9-*cis*-BC as shown above, this increased expression likely explains the lower 9-*cis*-BC levels in knockout as compared to WT mice.

## MOL #74732

We next investigated whether 9-*cis*-BC injection can result in 9-*cis*-retinoid production in *Bcmo1*<sup>-/-</sup> and *Bcmo2*<sup>-/-</sup> mice by analyzing their liver retinoid levels. Because retinoids mainly exist in the form of retinyl esters in the liver, we saponified liver samples and separated different retinol stereoisomers by HPLC (Fig. 4C). The major retinoid found was the all-*trans*-retinol stereoisomer with only trace amounts of 9-*cis*-retinol and 13-*cis*-retinol detected. In WT and *Bcd2*<sup>-/-</sup> mice, 9-*cis*-retinol levels were slightly higher than in *Bcmo1*<sup>-/-</sup> mice, but this difference did not achieve statistical significance. We also analyzed the retinoid composition in the eyes of these two knockout mouse strains (Fig. 4D). This analysis revealed that retinoid composition and levels in dark adapted eyes were comparable in the WT and knockout mice, with 11-*cis*-retinal and all-*trans*-retinyl esters identified as the major retinoids. Trace amounts of 9-*cis*-retinyl esters were present in the eyes of WT and *Bcd2*<sup>-/-</sup> mice but were absent in *Bcmo1*<sup>-/-</sup> mice. These experiments provide *in vivo* evidence that both carotenoid oxygenases can contribute to 9-*cis*-BC metabolism in mice. However, *i.p.* injection of 9-*cis*-BC did not result in significant production of 9-*cis*-retinoids in the eyes and livers of these animals.

### Metabolism of 9-*cis*-BC in *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice

Recently, it has been proposed that 9-*cis*-BC can improve vision in patients that suffer from RP (Rotenstreich et al., 2010). To test this proposal in animal models of this condition, we injected 6-week-old *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice *i.p.* with all-*trans*-BC, 9-*cis*-BC or vehicle (DMSO) control. At this early stage, mutant mice still display structurally intact photoreceptors (Wenzel et al., 2007). Importantly, we also included 9-*cis*-R-Ac injected mice as positive controls in this study. In this procedure, animals were injected daily *i.p.* with 0.2 mg of each compound dissolved in 60  $\mu$ l of DMSO. After five days of treatment, improvement of visual function was evaluated by ERG recording and 9-*cis*-retinal level in the eyes were quantified by normal phase HPLC (Fig. 5). In *Lrat*<sup>-/-</sup> mice, this analysis revealed that significant amounts of 9-*cis*-retinal were present in the eyes after the 9-*cis*-R-Ac injections. In contrast, 9-*cis*-BC administration did not

## MOL #74732

increase this compound in the eyes. As expected, all-*trans*-BC and vehicle control-injected mice showed the same negative result. In contrast, *Rpe65*<sup>-/-</sup> mice showed an increase of 9-*cis*-retinal after injections with either 9-*cis*-BC or 9-*cis*-R-Ac over levels found in vehicle only controls. No such increases were observed in mice that had undergone the all-*trans*-BC regimen. 9-*cis*-retinal levels in the eye after the 9-*cis*-R-Ac injection schedule was 25-fold higher than those of 9-*cis*-BC injected RPE65-deficient animals (Fig. 5A, B). ERG recording showed significant improvement of retinal function only in *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice treated with 9-*cis*-R-Ac. In contrast, administration with 9-*cis*-BC did not improve light sensitivity of the eyes (Fig. 5C, D). This result was consistent with the 9-*cis*-retinal level in *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice.

Thus, 9-*cis*-BC was not effective in promoting 9-*cis*-retinal production and restoring vision in the eyes of two mouse models of LCA. In contrast, 9-*cis*-R-Ac was efficiently used for 9-*cis*-retinal production and restored vision in the eyes of these mouse models.



**MOL #74732**

## **Discussion**

Double bonds in the carbon backbone of carotenoids and their retinoid derivatives can exist in *cis* and *trans*-configurations. In animals, 11-*cis*-retinal (or derivatives thereof) constitutes the chromophore of animal visual pigments (Wald, 1968). Additionally, 9-*cis*-retinoic acid can activate nuclear hormone receptors such as retinoic acid receptors and specifically the retinoid X receptors (Heyman et al., 1992). These are transcription factors that play important roles in processes as diverse as embryonic development, immunity and metabolic control. Moreover, 9-*cis*-retinal can bind the opsin moiety of visual pigments to form iso-rhodopsin. However, whether 9-*cis*-retinoids play a physiological role and how these compounds are synthesized from dietary precursors is controversial. Therefore, in this study we investigated 9-*cis*-BC metabolism and evaluated the effectiveness of 9-*cis*-BC support 9-*cis*-retinal production in the eyes of mouse models of RP.

### **9-*cis*-BC metabolism in mammals**

In gerbils, 9-*cis*-BC can be used as a dietary source of vitamin A but with only about 38% efficiency as compared to all-*trans*-BC (Deming et al., 2002). Additionally, this study showed that there was no increase of 9-*cis*-retinoids in the livers of mice upon 9-*cis*-BC supplementation. Our biochemical analyses and studies in animal models provide a mechanistic explanation for these observations. In the symmetrical cleavage reaction, BCMO1 converted 9-*cis*-BC into the all-*trans*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal stereoisomer in a molar ratio of 9:3:1 (Fig. 6). This finding contrasts with reports that 9-*cis*-BC is converted in one to one molar ratio to 9-*cis*-retinoids and all-*trans*-retinoids (Hebuterne et al., 1995; Wang et al., 1994). But it does agree with observations in cell free intestinal and liver extracts from rats that indicate a geometric composition of the cleavage products comparable to that we report with the

## MOL #74732

recombinant enzyme (Nagao and Olson, 1994). These findings suggest that BCMO1 has intrinsic isomerase activity similar to that recently demonstrated for structurally related enzymes in insects (Oberhauser et al., 2008; Voolstra et al., 2010). This enzyme converts all-*trans*-carotenoids to 11-*cis*-retinoids and all-*trans*-retinoids to support visual pigment production. This intrinsic isomerase activity is also in agreement with recent biochemical and structural analyses of this class of non-heme iron oxygenases revealing that the ferrous iron in the active site is accessible by a hydrophobic tunnel and a specific interaction with one half site of the carotenoid substrate (Kiser et al., 2009; Kloer et al., 2005). Measurements of the  $K_M$  value showed that BCMO1 has even a higher affinity for 9-*cis*-BC than all-*trans*-BC, indicating that the enzyme can interact with both ring sites of this substrate. Exclusion of the 9-*cis*-ring-site would result in a doubling of the  $K_M$  value as recently shown for the insect enzyme with asymmetric carotenoid substrates (Oberhauser et al., 2008). Several lines of evidence indicate that a carbocation intermediate is formed during reactions with these enzymes (Kiser et al., 2009; Poliakov et al., 2009). In this carbocation intermediate C-C bonds can undergo *trans*-to-*cis* and likely *cis*-to-*trans* isomerization as well. Thus, interaction of BCMO1 with the 9-*cis*-ring site should lead to an isomerization and the production of all-*trans*-retinal (Fig. 6). In contrast, interaction with the all-*trans*-site would lead to the production of 9-*cis*-retinal and all-*trans*-retinal. The latter mechanism could explain our observation that the all-*trans*-stereoisomer exists in 3-fold molar excess over the 9-*cis*-stereoisomer after enzymatic cleavage by BCMO1. Then there is an additional mechanism that reduces formation of 9-*cis*-retinoids from 9-*cis*-BC. BCDO2 preferentially removed the  $\beta$ -ionone ring site from the 9-*cis*-stereoisomeric site of BC resulting in the formation of all-*trans*- $\beta$ -10'-apocarotenal, which can then undergo a second cleavage reaction catalyzed by BCMO1 to form all-*trans*-retinal (Fig. 6). Indeed, we found a small increase of 9-*cis*-retinoids in the eyes and liver of *Bcd2*-deficient mice that overexpress BCMO1, thus indicating that some 9-*cis*-retinoids are produced in the absence of BCDO2. A subsequent cleavage of 9-*cis*-BC by both carotenoid oxygenases also contributes to the lower vitamin A-producing efficiency

## MOL #74732

of 9-*cis*-BC as compared to all-*trans*-BC. Furthermore, it provides an explanation for the finding that the stereoisomeric composition of liver retinoids was comparable after 9-*cis*-BC or all-*trans*-BC supplementation in WT animals (Deming et al., 2002). Conversion of 9-*cis*-BC by BCDO2 is also favored by the kinetics of 9-*cis*-BC conversion by BCMO1. BCMO1 showed a much lower conversion rate with the *cis*-stereoisomer, which also explains the observation that 9-*cis*-BC accumulates in tissues of WT mice. In knockout mice, this accumulation was less pronounced because of the compensatory increased expression of each remaining carotenoid oxygenase.

A critical question is whether our findings in mouse models can be translated into the human situation. In contrast to rodents, humans absorb a significant portion of dietary BC intact and display relatively high blood levels of this compound. Additionally, mechanisms exist to transport carotenoids to the eyes as demonstrated by the accumulation of macular pigments (zeaxanthin and lutein) in the *fovea lutea*. Thus, 9-*cis*-BC might be absorbed intact and transported to the eyes to be locally converted to 9-*cis*-retinoids. Molecular players for carotenoid metabolism are well conserved between rodents and humans (for review, (von Lintig, 2010). Recently, an individual with a heterozygotic mutation in *BCMO1* was described who evidenced both elevated plasma BC levels and low plasma retinol levels (Lindqvist et al., 2007). Additionally, the *BCDO2* gene is expressed in various human tissues (Lindqvist et al., 2005). For intestinal absorption, studies in humans showed that the all-*trans*-isomer is much better absorbed from the diet than the 9-*cis*-geometric states of BC (Gaziano et al., 1995; Stahl et al., 1993). Studies in humans also brought up the proposal that 9-*cis*-double bond of BC is isomerized to all-*trans*-double bond during absorption (Stahl et al., 1993). After [<sup>13</sup>C]-labeled 9-*cis*-BC supplementation, the resulting [<sup>13</sup>C]-retinoids existed mainly in the all-*trans*-configuration (You et al., 1996). Thus, 9-*cis*-BC is not well absorbed in humans and absorbed 9-*cis*-BC is mainly converted to all-*trans*-retinoids. The latter finding is likely explained by same enzymatic properties of human BCMO1 and BCDO2 as here described for their murine counterparts.

## MOL #74732

Collectively, our studies provide a mechanistic explanation for the observation that 9-*cis*-BC is mainly converted to all-*trans*-retinoids which can be further metabolized to all-*trans*-retinoic acid and 11-*cis*-retinal to support canonical retinoid action. These findings are in agreement with recent studies that evaluated the physiological role of 9-*cis*-retinoids in mammalian biology. Mouse studies indicate that only the all-*trans*-retinoic acid stereoisomer is required for normal development (Mic et al., 2003). Moreover, a recent analysis showed that 9-*cis*-retinoic acid is largely absent in mouse blood and tissues, except for the pancreas (Kane et al., 2010). Thus, there is no requirement for extensive 9-*cis*-retinoid production from dietary precursors under physiological conditions.

### Implications for the pharmacological use of 9-*cis*-BC and 9-*cis*-R-Ac

In pharmacological settings, agonists for RXR receptors, so called rexinoids, have been developed to fight cancer and metabolic diseases (de Lera et al., 2007). Additionally, 9-*cis*-R-Ac has been successfully used to restore vision and prevent retinal degeneration in animal models for RP (Palczewski, 2010). Recently, supplementation with *Dunaliella bardawil* extracts (containing 40-50% 9-*cis*-BC) have reportedly been beneficial for patients that suffer from *fundus albipunctatus*, a form of RP (Rotenstreich et al., 2010). Because unliganded opsin in chromophore-deficient photoreceptors provides a sink for 9-*cis*-retinal, we compared the relative potency of 9-*cis*-R-Ac and 9-*cis*-BC for this process. After a 5 day intervention with similar amounts of 9-*cis*-BC, all-*trans*-BC and 9-*cis*-R-Ac, we analyzed ocular retinoid composition of *Rpe65*<sup>-/-</sup> and *Lrat*<sup>-/-</sup> mice. In *Lrat*<sup>-/-</sup> mice, both BC stereoisomers failed to promote 9-*cis*-retinal production. But as expected, 9-*cis*-R-Ac proved successful in this process. In *Rpe65*<sup>-/-</sup> mice, a slight increase in 9-*cis*-retinal was observed upon 9-*cis*-BC injection that was absent in all-*trans*-BC supplemented animals. However, this increase was 25-fold lower than observed after 9-*cis*-R-Ac injections and improvement of retinal function was not obvious in ERG responses obtained from *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice after 9-*cis*-BC administration. This pronounced difference is likely

## MOL #74732

explained by our finding that only a portion of 9-*cis*-BC is metabolized to 9-*cis*-retinoids because of *cis*-to-*trans* isomerization by BCMO1 and subsequent removal of the 9-*cis*- $\beta$ -ionone ring site by BCDO2. Nevertheless, this finding can explain why the eyes of *Rpe65*<sup>-/-</sup> can contain some 9-*cis*-retinal. The absence of 9-*cis*-retinal in photoreceptors of LRAT mice indicates that retinyl ester formation is required to accumulate 9-*cis*-retinoids produced from 9-*cis*-BC in the eyes. We previously have shown that LRAT acts downstream of the retinoid transporter STRA6 to enhance the uptake of retinoids bound to the serum retinol binding protein (Isken et al., 2008). This dependency can obviously be bypassed by 9-*cis*-R-Ac. However, there also is less 9-*cis*-retinal accumulation in the eyes of *Lrat*<sup>-/-</sup> mice as compared to *Rpe65*<sup>-/-</sup> mice after 9-*cis*-R-Ac injections. Thus, 9-*cis*-BC as compared to 9-*cis*-R-Ac is a poor source for 9-*cis*-retinal in mouse models of LCA and because of poor absorption and conversion to all-*trans*-retinoids likely in humans as well.

**MOL #74732**

## ACKNOWLEDGMENTS

We would like to thank Dr. Zhiqian Dong for expert handling of mice. We also thank Dr. L.T. Webster, Jr. for critical comments on the manuscript. KP is John H. Hord Professor of Pharmacology.

### Authorship Contributions:

*Participated in research design:* Tadao Maeda, Krzysztof Palczewski, and Johannes von Lintig

*Conducted experiments:* Tadao Maeda, Lindsay Perusek, Jaume Amangua, Darwin Babino

*Contributed new reagents or analytic tools:* not applicable

*Performed data analysis:* Tadao Maeda, Krzysztof Palczewski, and Johannes von Lintig

*Wrote or contributed to the writing of the manuscript:* Johannes von Lintig and Krzysztof Palczewski.

MOL #74732

## References

- Amengual J, Gouranton E, van Helden YG, Hessel S, Ribot J, Kramer E, Kiec-Wilk B, Razny U, Lietz G, Wyss A, Dembinska-Kiec A, Palou A, Keijer J, Landrier JF, Bonet ML and von Lintig J (2011a) Beta-Carotene Reduces Body Adiposity of Mice via BCMO1. *PLoS one* **6**(6):e20644.
- Amengual J, Lobo GP, Golczak M, Li HN, Klimova T, Hoppel CL, Wyss A, Palczewski K and von Lintig J (2011b) A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. *FASEB J* **25**(3):948-959.
- Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W and Palczewski K (2004) Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. *J Biol Chem* **279**(11):10422-10432.
- Batten ML, Imanishi Y, Tu DC, Doan T, Zhu L, Pang J, Glushakova L, Moise AR, Baehr W, Van Gelder RN, Hauswirth WW, Rieke F and Palczewski K (2005) Pharmacological and rAAV gene therapy rescue of visual functions in a blind mouse model of Leber congenital amaurosis. *PLoS Med* **2**(11):e333.
- Ben-Amotz A, Lers A and Avron M (1988) Stereoisomers of beta-Carotene and Phytoene in the Alga *Dunaliella bardawil*. *Plant Physiol* **86**(4):1286-1291.
- Chichili GR, Nohr D, Schaffer M, von Lintig J and Biesalski HK (2005) beta-Carotene conversion into vitamin A in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* **46**(10):3562-3569.
- de Lera AR, Bourguet W, Altucci L and Gronemeyer H (2007) Design of selective nuclear receptor modulators: RAR and RXR as a case study. *Nat Rev Drug Discov* **6**(10):811-820.
- Deming DM, Baker DH and Erdman JW, Jr. (2002) The relative vitamin A value of 9-cis beta-carotene is less and that of 13-cis beta-carotene may be greater than the accepted 50% that of all-trans beta-carotene in gerbils. *J Nutr* **132**(9):2709-2712.
- Demmig-Adams B and Adams WW, 3rd (2002) Antioxidants in photosynthesis and human nutrition. *Science* **298**(5601):2149-2153.
- den Hollander AI, Black A, Bennett J and Cremers FP (2011) Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies. *J Clin Invest* **120**(9):3042-3053.
- den Hollander AI, Roepman R, Koenekoop RK and Cremers FP (2008) Leber congenital amaurosis: genes, proteins and disease mechanisms. *Prog Retin Eye Res* **27**(4):391-419.
- Fan J, Rohrer B, Moiseyev G, Ma JX and Crouch RK (2003) Isorhodopsin rather than rhodopsin mediates rod function in RPE65 knock-out mice. *Proc Natl Acad Sci U S A* **100**(23):13662-13667.
- Fleisch VC and Neuhauss SC (2010) Parallel visual cycles in the zebrafish retina. *Prog Retin Eye Res* **29**(6):476-486.
- Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, Ridker PM, Frei B, Hennekens CH and Krinsky NI (1995) Discrimination in absorption or transport of beta-carotene isomers after oral supplementation with either all-trans- or 9-cis-beta-carotene. *Am J Clin Nutr* **61**(6):1248-1252.
- Hebuterne X, Wang XD, Johnson EJ, Krinsky NI and Russell RM (1995) Intestinal absorption and metabolism of 9-cis-beta-carotene in vivo: biosynthesis of 9-cis-retinoic acid. *J Lipid Res* **36**(6):1264-1273.

**MOL #74732**

- Hessel S, Eichinger A, Isken A, Amengual J, Hunzelmann S, Hoeller U, Elste V, Hunziker W, Goralczyk R, Oberhauser V, von Lintig J and Wyss A (2007) CMO1 deficiency abolishes vitamin A production from beta-carotene and alters lipid metabolism in mice. *J Biol Chem* **282**(46):33553-33561.
- Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM and Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**(2):397-406.
- Isken A, Golczak M, Oberhauser V, Hunzelmann S, Driever W, Imanishi Y, Palczewski K and von Lintig J (2008) RBP4 disrupts vitamin A uptake homeostasis in a STRA6-deficient animal model for Matthew-Wood syndrome. *Cell Metab* **7**(3):258-268.
- Kane MA, Folias AE, Pingitore A, Perri M, Obrochta KM, Krois CR, Cione E, Ryu JY and Napoli JL (2010) Identification of 9-cis-retinoic acid as a pancreas-specific autacoid that attenuates glucose-stimulated insulin secretion. *Proc Natl Acad Sci U S A* **107**(50):21884-21889.
- Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO, Breithaupt DE and von Lintig J (2001) Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J Biol Chem* **276**(17):14110-14116.
- Kim YK, Wassef L, Chung S, Jiang H, Wyss A, Blaner WS and Quadro L (2011) beta-Carotene and its cleavage enzyme beta-carotene-15,15'-oxygenase (CMOI) affect retinoid metabolism in developing tissues. *FASEB J* **25**(5):1641-1652.
- Kiser PD, Golczak M, Lodowski DT, Chance MR and Palczewski K (2009) Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. *Proc Natl Acad Sci U S A* **106**(41):17325-17330.
- Kloer DP, Ruch S, Al-Babili S, Beyer P and Schulz GE (2005) The structure of a retinal-forming carotenoid oxygenase. *Science* **308**(5719):267-269.
- Lindqvist A, He YG and Andersson S (2005) Cell type-specific expression of beta-carotene 9',10'-monooxygenase in human tissues. *J Histochem Cytochem* **53**(11):1403-1412.
- Lindqvist A, Sharvill J, Sharvill DE and Andersson S (2007) Loss-of-function mutation in carotenoid 15,15'-monooxygenase identified in a patient with hypercarotenemia and hypovitaminosis A. *J Nutr* **137**(11):2346-2350.
- Lobo G, Hessel S, Eichinger A, Noy N, Moise AR, Wyss A, Palczewski K and von Lintig J (2010a) ISX is a retinoic acid sensitive gatekeeper that controls intestinal beta-carotene absorption and vitamin A production. *FASEB J* 2010 Jan 8 [Epub ahead of print].
- Lobo GP, Hessel S, Eichinger A, Noy N, Moise AR, Wyss A, Palczewski K and von Lintig J (2010b) ISX is a retinoic acid-sensitive gatekeeper that controls intestinal beta,beta-carotene absorption and vitamin A production. *FASEB J* **24**(6):1656-1666.
- Maeda T, Maeda A, Casadesus G, Palczewski K and Margaron P (2009) Evaluation of 9-cis-retinyl acetate therapy in Rpe65<sup>-/-</sup> mice. *Invest Ophthalmol Vis Sci* **50**(9):4368-4378.
- Mic FA, Molotkov A, Benbrook DM and Duester G (2003) Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc Natl Acad Sci U S A* **100**(12):7135-7140.
- Nagao A and Olson JA (1994) Enzymatic formation of 9-cis, 13-cis, and all-trans retinals from isomers of beta-carotene. *FASEB J* **8**(12):968-973.
- Oberhauser V, Voolstra O, Bangert A, von Lintig J and Vogt K (2008) NinaB combines carotenoid oxygenase and retinoid isomerase activity in a single polypeptide. *Proc Natl Acad Sci U S A* **105**(48):19000-19005.
- Obulesu M, Dowlathabad MR and Bramhachari PV (2011) Carotenoids and Alzheimer's Disease: An insight into therapeutic role of retinoids in animal models. *Neurochem Int*.
- Palczewski K (2006) G protein-coupled receptor rhodopsin. *Annu Rev Biochem* **75**:743-767.
- Palczewski K (2010) Retinoids for treatment of retinal diseases. *Trends Pharmacol Sci* **31**(6):284-295.



**MOL #74732**

- Parker RS (1996) Absorption, metabolism, and transport of carotenoids. *FASEB J* **10**(5):542-551.
- Poliakov E, Gentleman S, Chander P, Cunningham FX, Jr., Grigorenko BL, Nemuhin AV and Redmond TM (2009) Biochemical evidence for the tyrosine involvement in cationic intermediate stabilization in mouse beta-carotene 15, 15'-monooxygenase. *BMC Biochem* **10**:31.
- Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK and Pfeifer K (1998) Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet* **20**(4):344-351.
- Rotenstreich Y, Harats D, Shaish A, Pras E and Belkin M (2010) Treatment of a retinal dystrophy, fundus albipunctatus, with oral 9-cis- $\beta$ -carotene. *Br J Ophthalmol* **94**(5):616-621.
- Stahl W, Schwarz W and Sies H (1993) Human serum concentrations of all-trans beta- and alpha-carotene but not 9-cis beta-carotene increase upon ingestion of a natural isomer mixture obtained from *Dunaliella salina* (Betatene). *J Nutr* **123**(5):847-851.
- Thompson DA and Gal A (2003) Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. *Prog Retin Eye Res* **22**(5):683-703.
- Travis GH, Golczak M, Moise AR and Palczewski K (2007) Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. *Annu Rev Pharmacol Toxicol* **47**:469-512.
- Van Hooser JP, Aleman TS, He YG, Cideciyan AV, Kuksa V, Pittler SJ, Stone EM, Jacobson SG and Palczewski K (2000) Rapid restoration of visual pigment and function with oral retinoid in a mouse model of childhood blindness. *Proc Natl Acad Sci U S A* **97**(15):8623-8628.
- von Lintig J (2010) Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. *Annu Rev Nutr* **30**:35-56.
- von Lintig J, Kiser PD, Golczak M and Palczewski K (2010) The biochemical and structural basis for trans-to-cis isomerization of retinoids in the chemistry of vision. *Trends Biochem Sci* **35**(7):400-410.
- Voolstra O, Oberhauser V, Sumser E, Meyer NE, Maguire ME, Huber A and von Lintig J (2010) NinaB is essential for *Drosophila* vision but induces retinal degeneration in opsin-deficient photoreceptors. *J Biol Chem* **285**(3):2130-2139.
- Wald G (1968) Molecular basis of visual excitation. *Science* **162**(850):230-239.
- Wang JS and Kefalov VJ (2011) The cone-specific visual cycle. *Prog Retin Eye Res* **30**(2):115-128.
- Wang XD, Krinsky NI, Benotti PN and Russell RM (1994) Biosynthesis of 9-cis-retinoic acid from 9-cis-beta-carotene in human intestinal mucosa in vitro. *Arch Biochem Biophys* **313**(1):150-155.
- Wenzel A, von Lintig J, Oberhauser V, Tanimoto N, Grimm C and Seeliger MW (2007) RPE65 is essential for the function of cone photoreceptors in NRL-deficient mice. *Invest Ophthalmol Vis Sci* **48**(2):534-542.
- Yan W, Jang GF, Haeseleer F, Esumi N, Chang J, Kerrigan M, Campochiaro M, Campochiaro P, Palczewski K and Zack DJ (2001) Cloning and characterization of a human beta,beta-carotene-15,15'-dioxygenase that is highly expressed in the retinal pigment epithelium. *Genomics* **72**(2):193-202.
- You CS, Parker RS, Goodman KJ, Swanson JE and Corso TN (1996) Evidence of cis-trans isomerization of 9-cis-beta-carotene during absorption in humans. *Am J Clin Nutr* **64**(2):177-183.

**MOL #74732**

**Footnotes:** This research was supported in part by grants from the National Institute of Health [EY019641, EY009339, EY021126, EY019880, EY020551 and P30 EY11373], the Research to Prevent Blindness Foundation and the Foundation Fighting Blindness.

University of Washington and QLT Inc. may commercialize some of the technology described in this work. KP and TM are consultants for QLT Inc.

MOL #74732

## Figures Legends

### Figure 1. Products of recombinant murine BCMO1 enzymatic activity.

Protein extracts of recombinant murine BCMO1 were incubated in the presence of 20  $\mu$ M all-*trans*-BC or 9-*cis*-BC. After 5 min, lipids were extracted and separated by normal phase HPLC. (A) HPLC monitored at 360 nm. Upon incubation of BCMO1 with all-*trans*-BC significant amounts of all-*trans*-retinal were produced (solid line). The identity of this compound was verified by co-chromatography with an authentic standard (dotted line). Note that all-*trans*-retinal was converted to the corresponding *syn*- and *anti*-oxime forms during extraction. Peak 1, all-*trans*-retinal-oxime (*syn*); peak 2, all-*trans*-retinal-oxime (*anti*). (B) Spectra of all-*trans*-retinal-oxime (*syn*) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line). (C) HPLC monitored at 360 nm. Upon incubation of BCMO1 with 9-*cis*-BC different retinal stereoisomers became detectable (solid line). The identity of these compounds was verified by co-chromatography with authentic standards for all-*trans*-retinal (dotted line) and 9-*cis*-retinal (dashed line). Note that different retinal stereoisomers were converted to the corresponding *syn*- and *anti*-oxime forms during extraction. Peak 1, all-*trans*-retinal-oxime (*syn*); peak 2, 9-*cis*-retinal-oxime (*syn*); peak 3, 13-*cis*-retinal-oxime (*syn*); peak 4, 13-*cis*-retinal-oxime (*anti*); peak 5, 9-*cis*-retinal-oxime (*anti*); and peak 6, all-*trans*-retinal-oxime (*anti*). (D) (left) Spectra of all-*trans*-retinal-oxime (*syn*) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line). (E) Spectral characteristics of 9-*cis*-retinal-oxime (*syn*) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line).

### Figure 2. Kinetics of the conversion of all-*trans*-BC and 9-*cis*-BC by murine BCMO1.

## MOL #74732

Protein extract with recombinant murine BCMO1 was incubated in the presence of 20  $\mu$ M all-*trans*-BC or 9-*cis*-BC. Reactions were stopped after different time intervals; lipids were extracted and subjected to HPLC analysis. Each value shown represents the average of three independent experiments. (A) all-*trans*-BC. (B) 9-*cis*-BC. Insets show the amounts of retinal stereoisomers produced per minute by the same enzyme preparation. Values represent the means  $\pm$  SD from three independent enzymatic assays. (C, D) Lineweaver-Burk blots of reactions with (C) all-*trans*-BC and (D) 9-*cis*-BC. Each value represents the mean  $\pm$  SD of three independent enzymatic assays.

### Figure 3. Reactions of BCDO2 with all-*trans*-BC and 9-*cis*-BC.

The same BCDO2 enzyme preparation was incubated in the presence of 20  $\mu$ M all-*trans*-BC or 9-*cis*-BC. Reactions were stopped after 5 min and lipids were extracted and separated by HPLC. Note that the apocarotenoids were converted to the corresponding oximes during extraction. (A) HPLC at 420 nm. Lower trace, 9-*cis*-BC without incubation; dashed trace, all-*trans*-BC; dashed-dotted trace, 9-*cis*-BC; dotted trace; all-*trans*- $\beta$ -10'-apocarotenal standard. peak 1, 10'- $\beta$ -apocarotenal-oxime (*syn*); peak 2, 10'- $\beta$ -apocarotenal-oxime (*anti*); peak 3, 9-*cis*-10'- $\beta$ -apocarotenal. (B) Conversion rates of all-*trans*-BC and 9-*cis*-BC. Values represent the mean  $\pm$  SD of three independent enzymatic assays. (C) Spectral characteristics of peak 1, 10'- $\beta$ -apocarotenal-oxime (*syn*) (left) produced by the enzymatic reaction (solid line) and from the authentic standard (dotted line) and peak 3, 9-*cis*-10'- $\beta$ -apocarotenal.

### Figure 4. Retinoid profiles in mouse eyes and liver after administration of $\beta$ , $\beta$ -carotene isomers.

*Bcmo1*<sup>-/-</sup>, *Bcd2*<sup>-/-</sup> and WT mice were treated with 9-*cis*-BC at 1.0 mg/mouse via *i.p.* injection over the course of five consecutive days. Retinoid profiles in the eyes and liver were evaluated

## MOL #74732

by normal phase silica column chromatography. Trace levels of 9-*cis*-retinyl esters detected in WT and *Bcdo2*<sup>-/-</sup> mouse eyes were not observed in *Bcmo1*<sup>-/-</sup> or WT mice treated with DMSO. (A). Significant amounts of β,β-carotenes were detected in WT liver whereas their levels were lower in *Bcmo1*<sup>-/-</sup> and *Bcdo2*<sup>-/-</sup> mice ( < 5% compared with WT) after 9-*cis*-BC treatment. (B). qRT-PCR analysis of BCMO1 and BCDO2 expression in livers of indicated mouse strains values are represented as fold changes as compared to WT and are normalized to levels of 18S rRNA. Error bars represent ± S.D., n = 5 per genotype. Two tailed student's *t*-test, \* *p* ≤ 0.01. ND, not detectable (C) Retinoid composition of the livers of different mouse strains upon 9-*cis*-BC injections. (D) Retinoid composition of the eyes upon 5 daily 9-*cis*-BC injections. Three mice representing each genotype were treated.

**Figure 5.** Eye retinoid profile and retinal function after administration of BC isomers and 9-*cis*-R-Ac.

Purified BC isomers and 9-*cis*-R-Ac at 1.0 mg/mouse (total) in DMSO was administrated over the course of five consecutive days via *i.p* injection to *Rpe65*<sup>-/-</sup> and *Lrat*<sup>-/-</sup> mice maintained on a low vitamin A diet. After 5 days without treatment or light exposure, eye retinoid profiles were determined by normal phase silica column chromatography and improvement of retinal function was evaluated by ERG. Representative chromatograms from each group of mice are shown (A). Visual pigment regeneration was evaluated by determining levels of 9-*cis*-retinal in the eye which reflect isorhodopsin levels in the retina. 9-*cis*-Retinal was detected in eyes of *Lrat*-deficient and *Rpe65*-deficient mice treated with 9-*cis*-R-Ac (A, black arrows) whereas significantly lower levels of 9-*cis*-retinal were found in eyes of 9-*cis*-BC-treated *Rpe65*-deficient animals but not of *Lrat*-deficient animals (A and B, less than 4.0 % compared with 9-*cis*-R-Ac treatment). Trace level amounts of 9-*cis*-retinal was detected in the eyes of either mutant strain treated with all-*trans*-BC or DMSO (A and B). Serial ERG responses in RPE65-deficient and

## MOL #74732

LRAT-deficient mice after treatment with each compound were compared (C). Amplitudes of functional b-wave versus intensity of stimuli were plotted (D). Apparent improvement of ERG responses were only observed in 9-*cis*-R-Ac treated mice. ND, not detectable. For RPE65-deficient 6, 7, 7 and 2 mice were treated with DMSO, all-*trans*-BC, 9-*cis*-BC and 9-*cis*-R-Ac respectively. Likewise 3, 3, 4 and 4 LRAT-deficient mice were treated with all-*trans*-BC, 9-*cis*-BC and 9-*cis*-R-Ac respectively. ERG responses were obtained from 2 mice (4 eyes) of RPE65-deficient and LRAT-deficient mice which were treated with each compound and separately prepared from retinoid analyses.

### **Figure 6.** Model of 9-*cis*- $\beta$ , $\beta$ -carotene metabolism in mice.

9-*cis*-BC can be metabolized by the two different carotenoid-oxygenases BCMO1 (1) and BCDO2 (2). The percentage of occurrence of the different cleavage product stereoisomers was determined by enzymatic assays with recombinant proteins. For BCMO1, the discrepancy of these values from the theoretical value (50%) indicates that the enzyme can interact with both  $\beta$ -ionone ring sites and that the enzyme possesses intrinsic isomerase activity when interacting with the 9-*cis*-ring site. For BCDO2, the discrepancy of these values from the theoretical value (50%) indicates that the enzyme preferentially removes the 9-*cis*-ring site. The cleavage product all-*trans*- $\beta$ -10'-apocarotenal can be further metabolized by BCMO1. The second cleavage product 9-*cis*- $\beta$ -10'-apocarotenal also is likely metabolized by BCMO1. In conclusion, these findings provide an explanation for the lack of production of significant amounts of 9-*cis*-retinoids from 9-*cis*-BC in different mouse models. a, 9-*cis*-BC; b, 9-*cis*-retinal; c, all-*trans*-retinal; d, all-*trans*- $\beta$ -10'-apocarotenal; e, 9-*cis*- $\beta$ -10'-apocarotenal, f,  $\beta$ -ionone; ??, not experimentally tested.

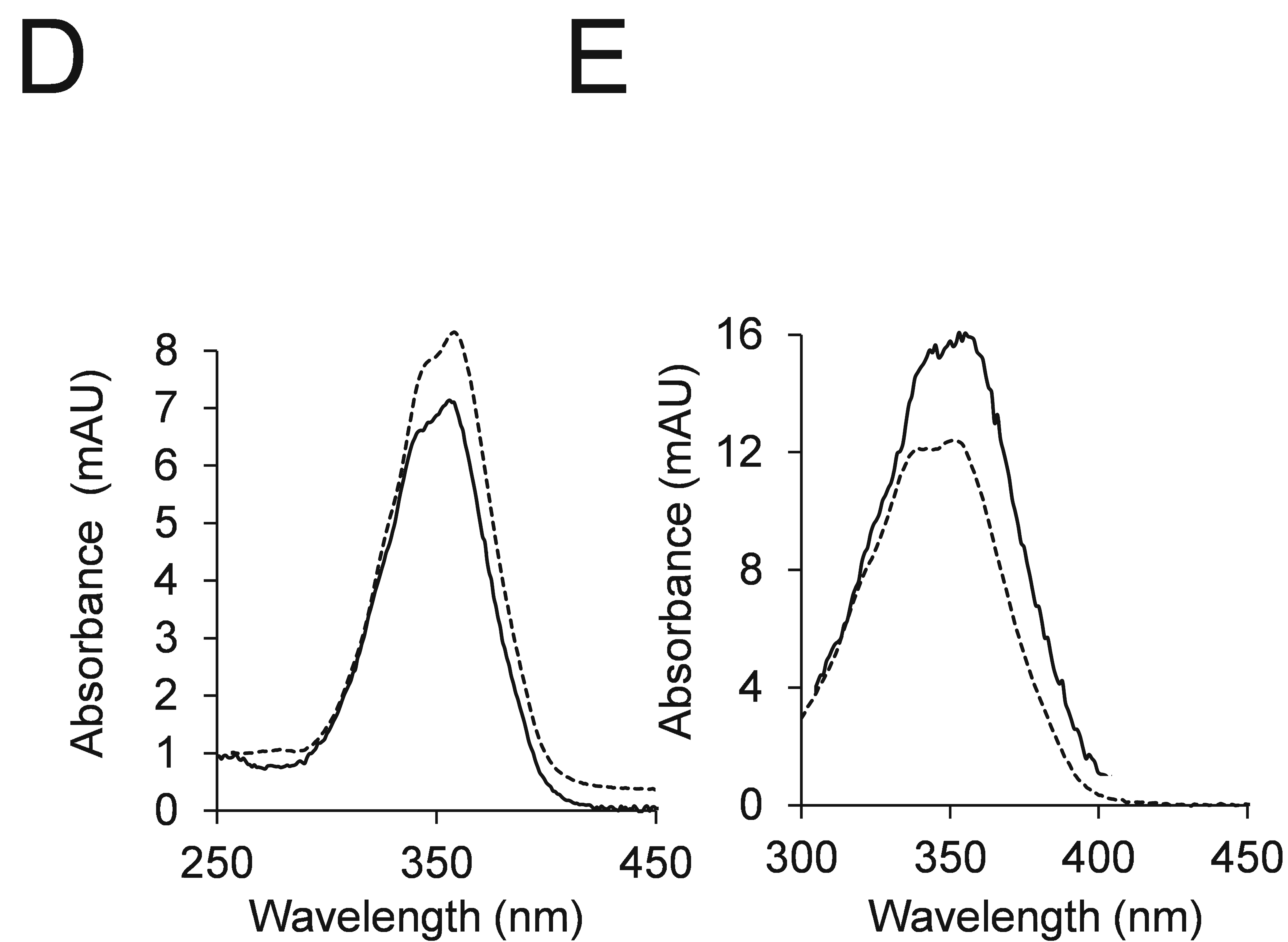
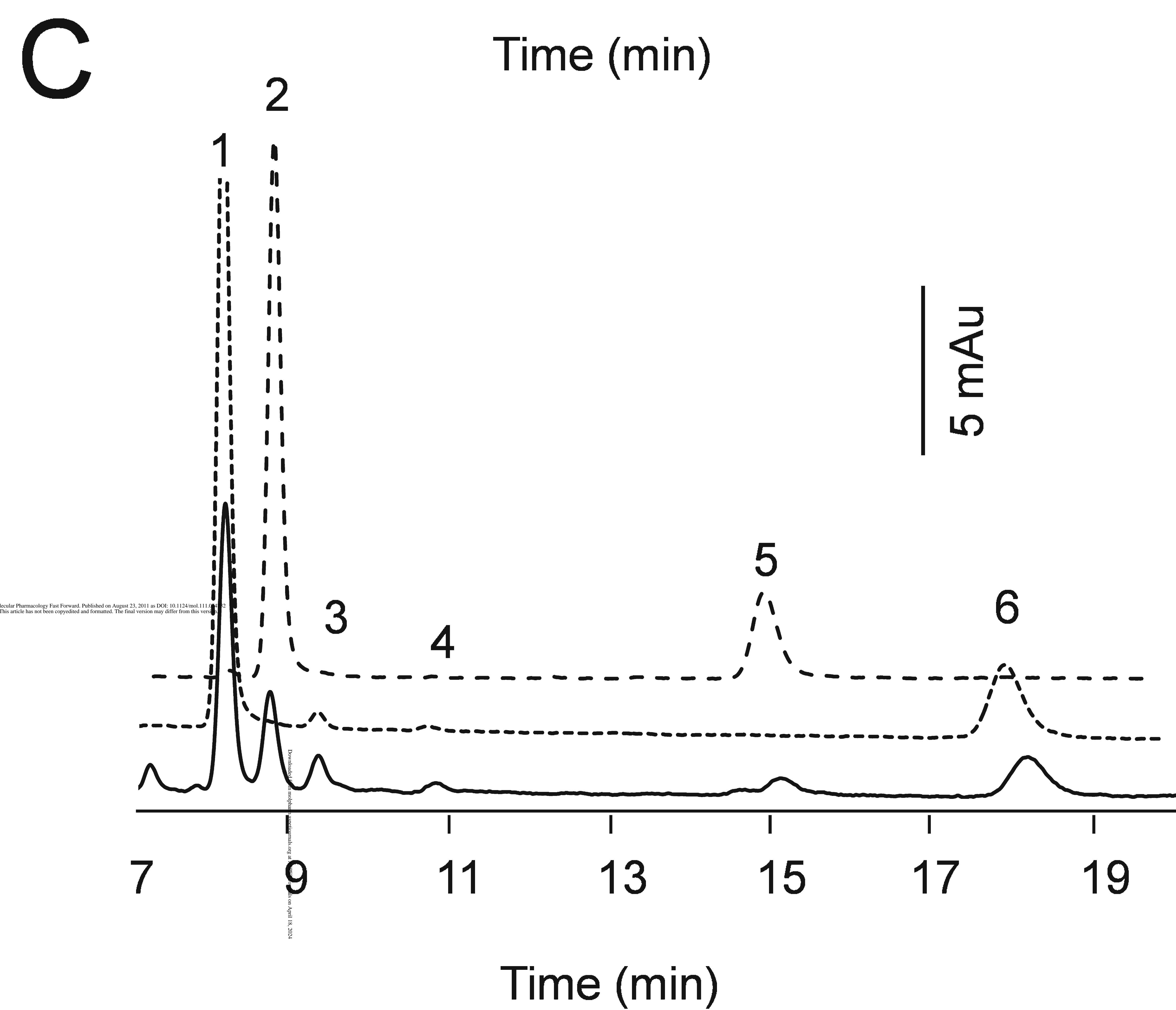
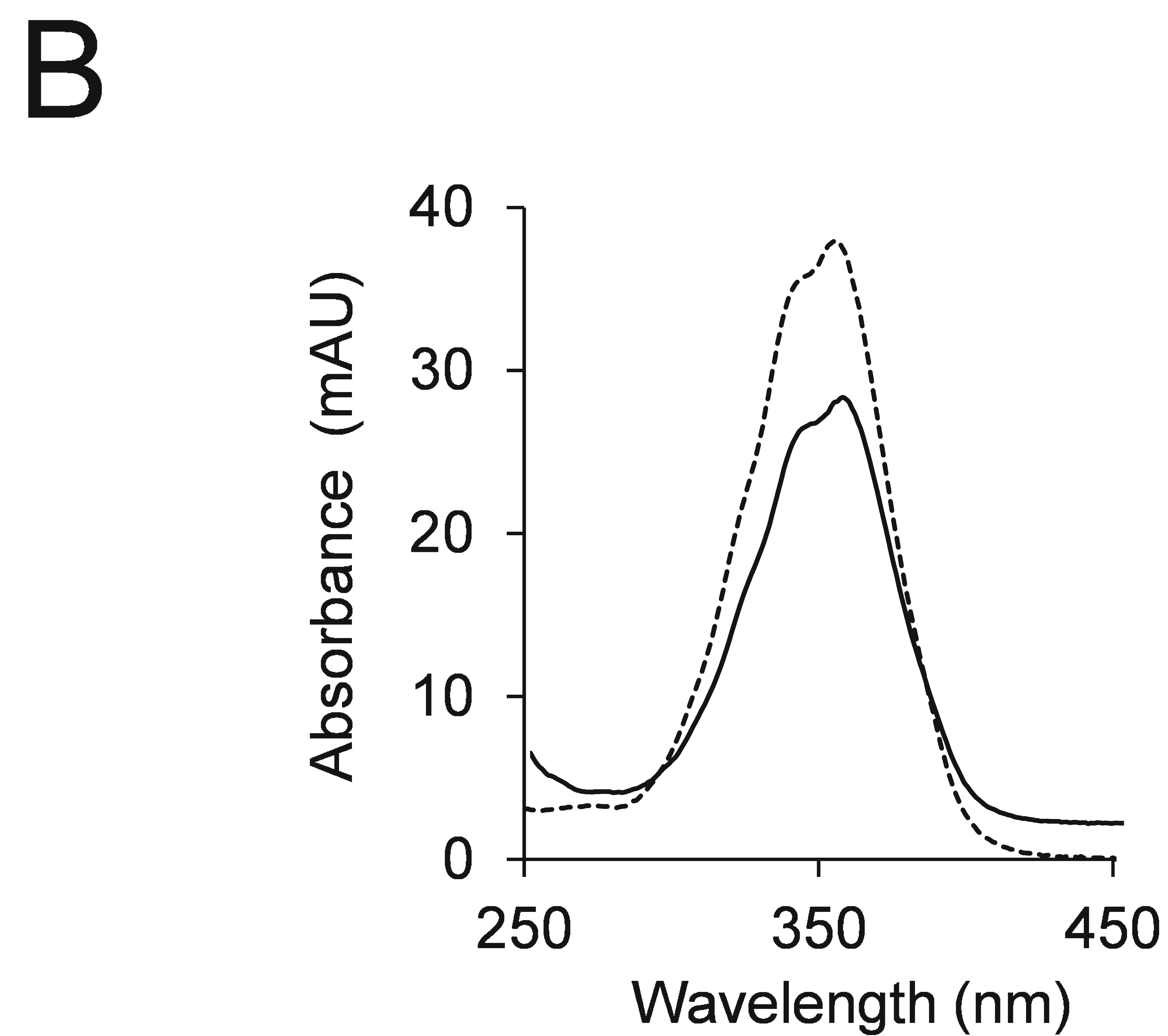
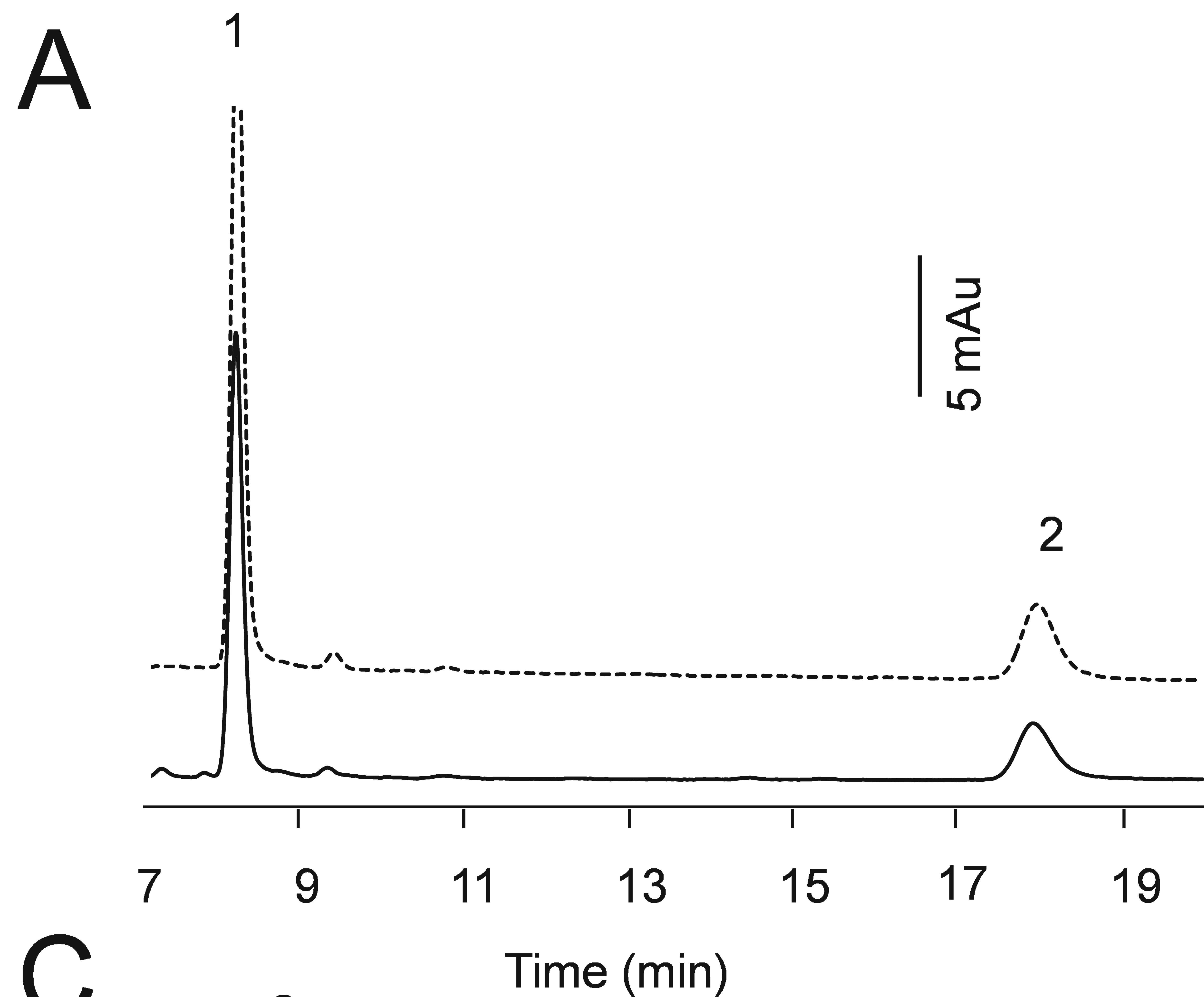


Fig 2

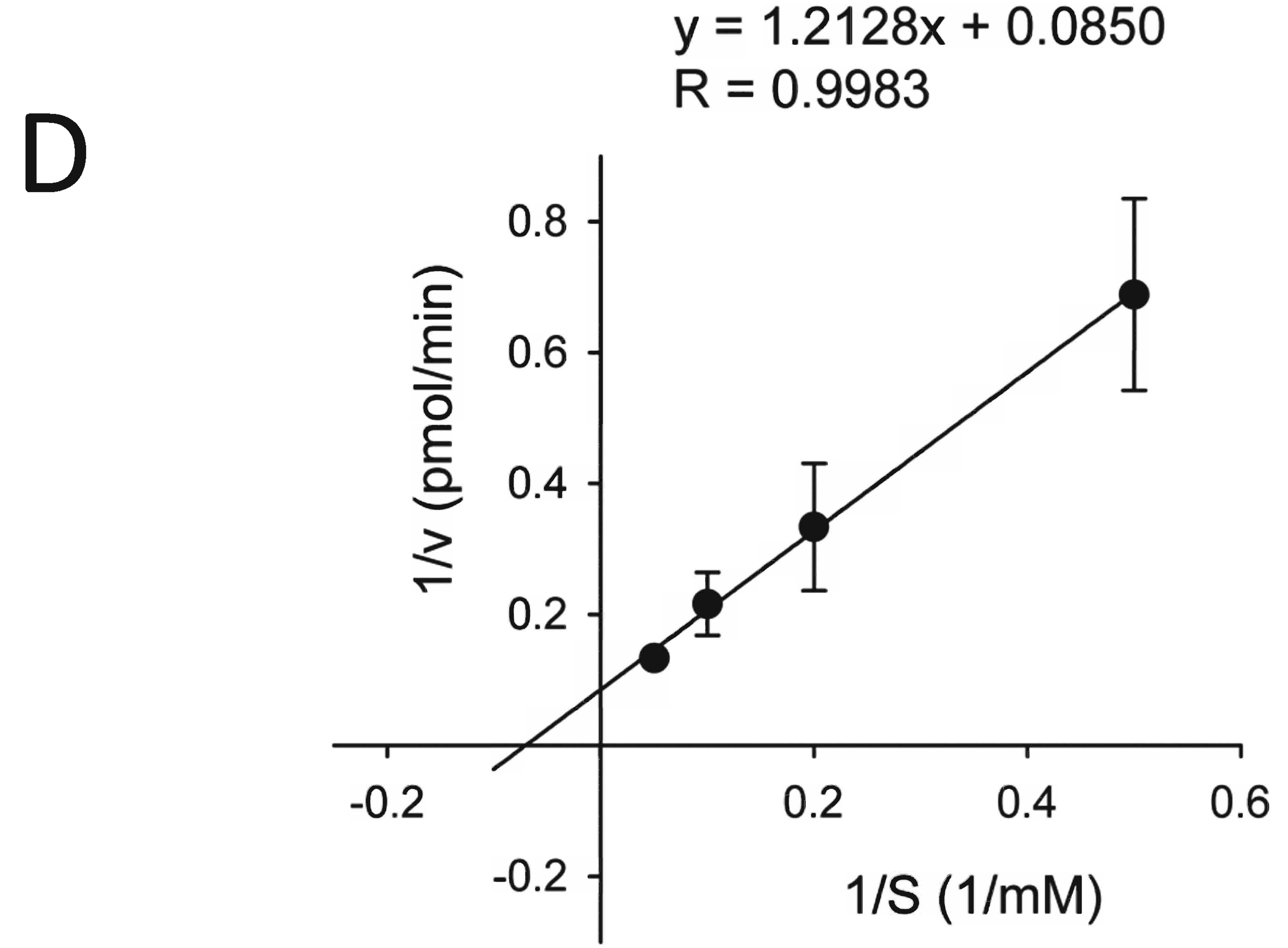
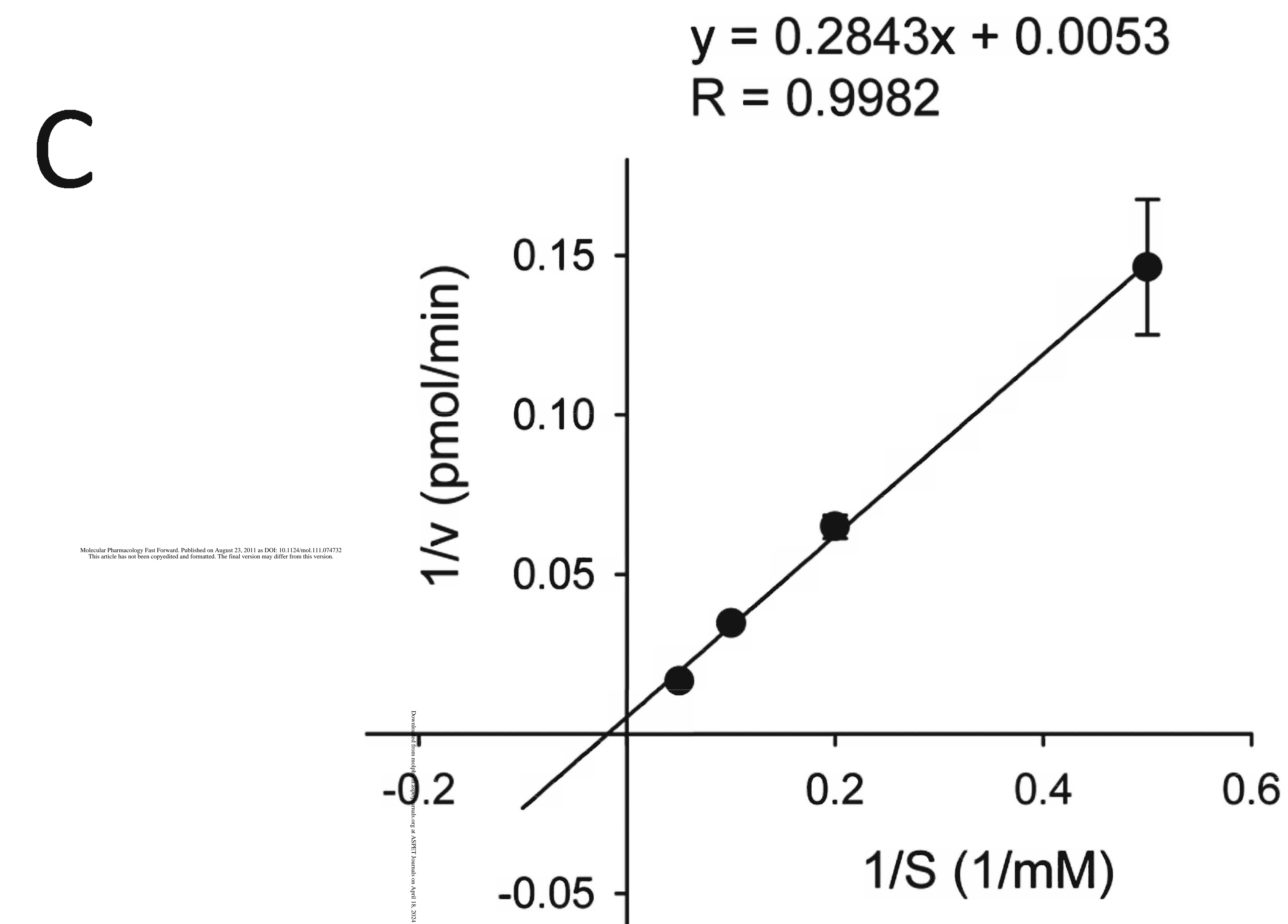
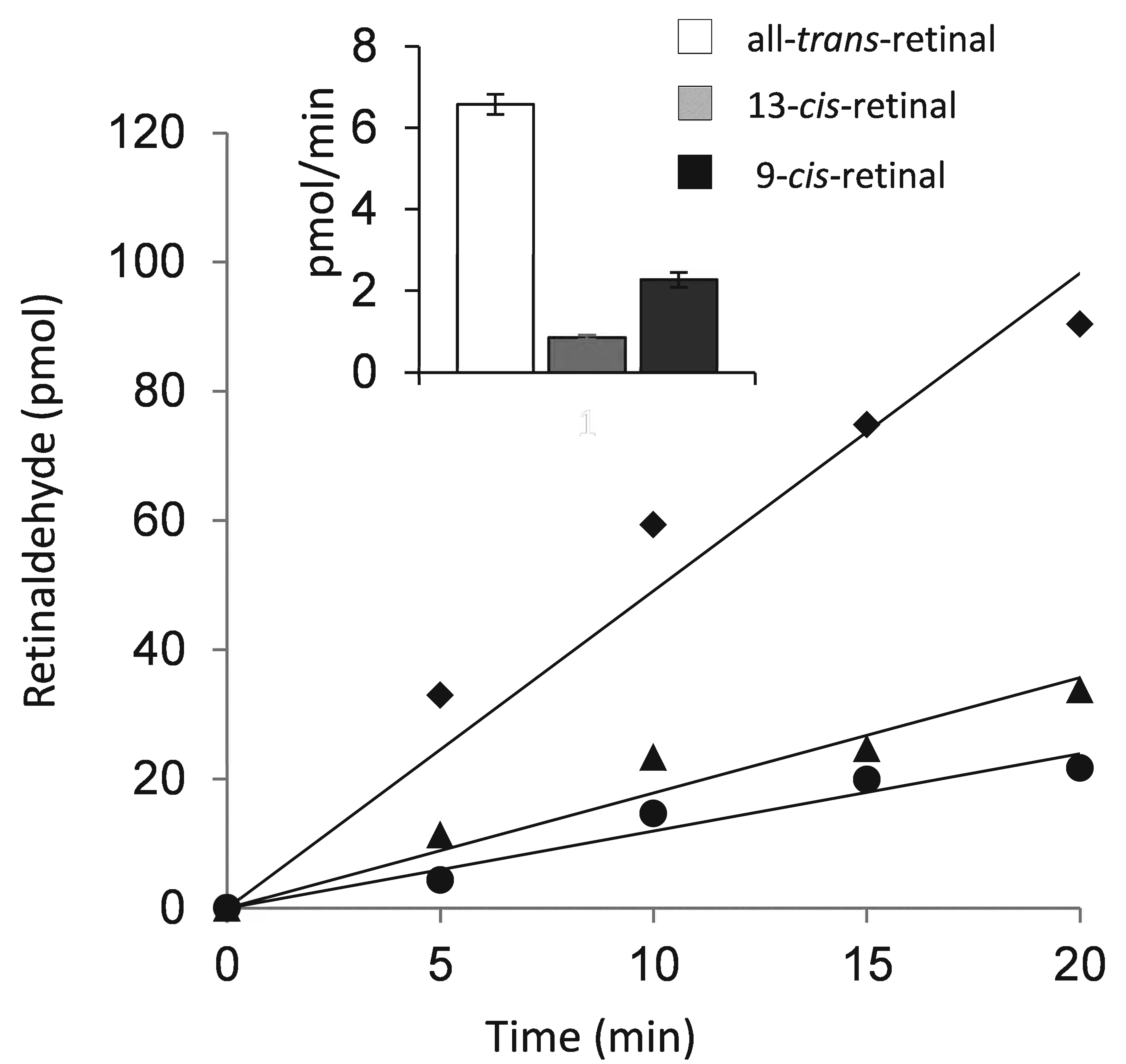
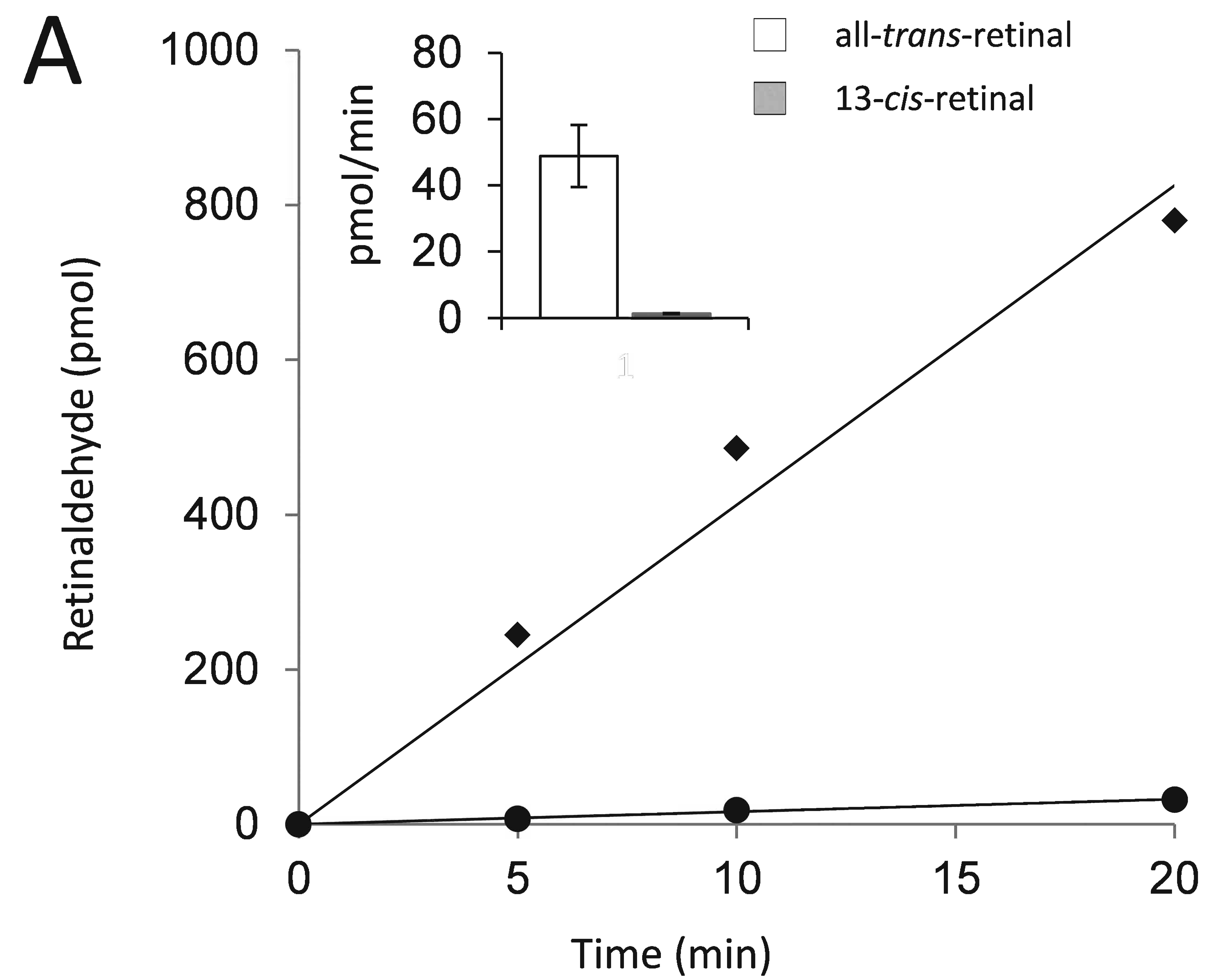




Fig 3

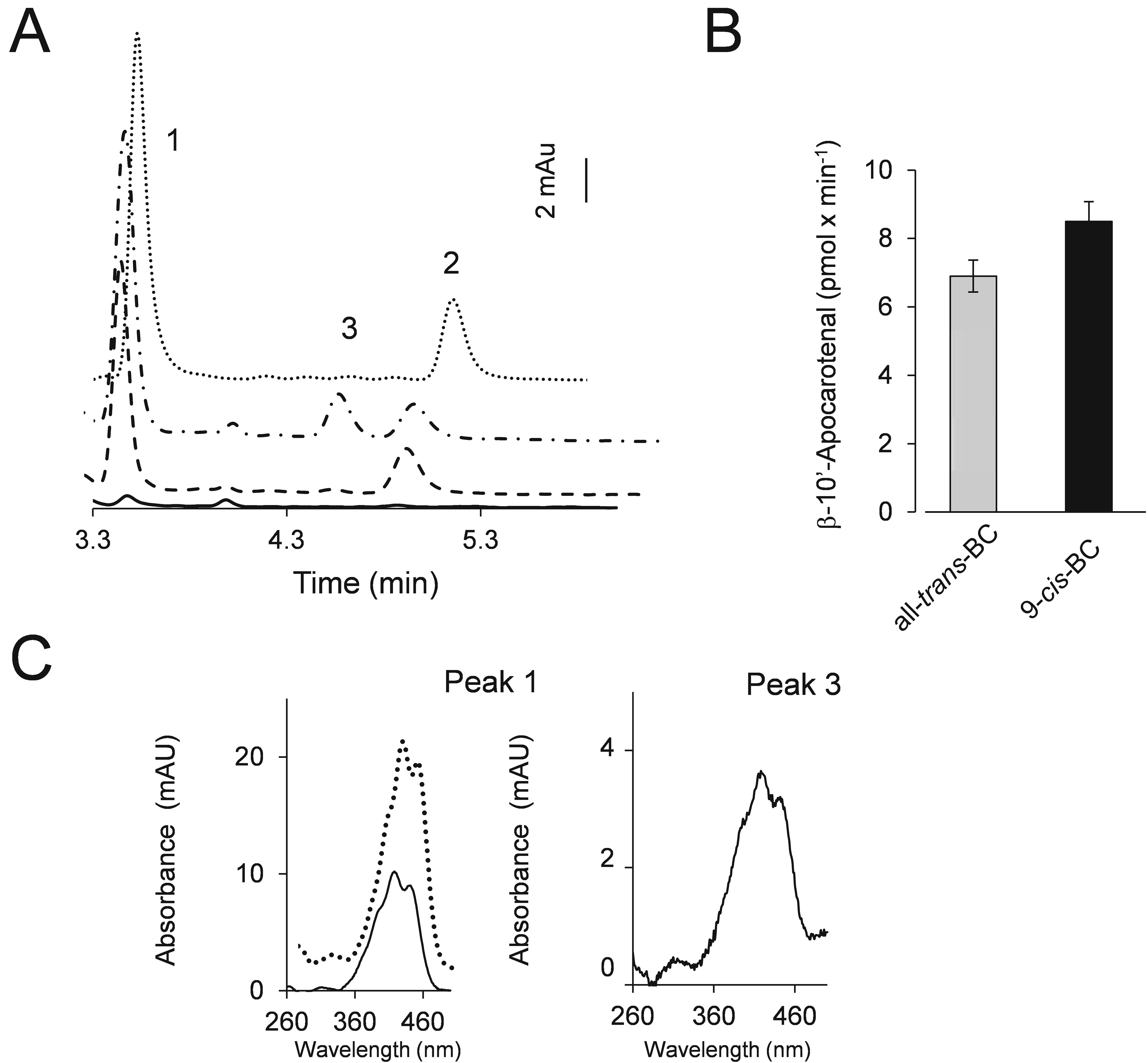
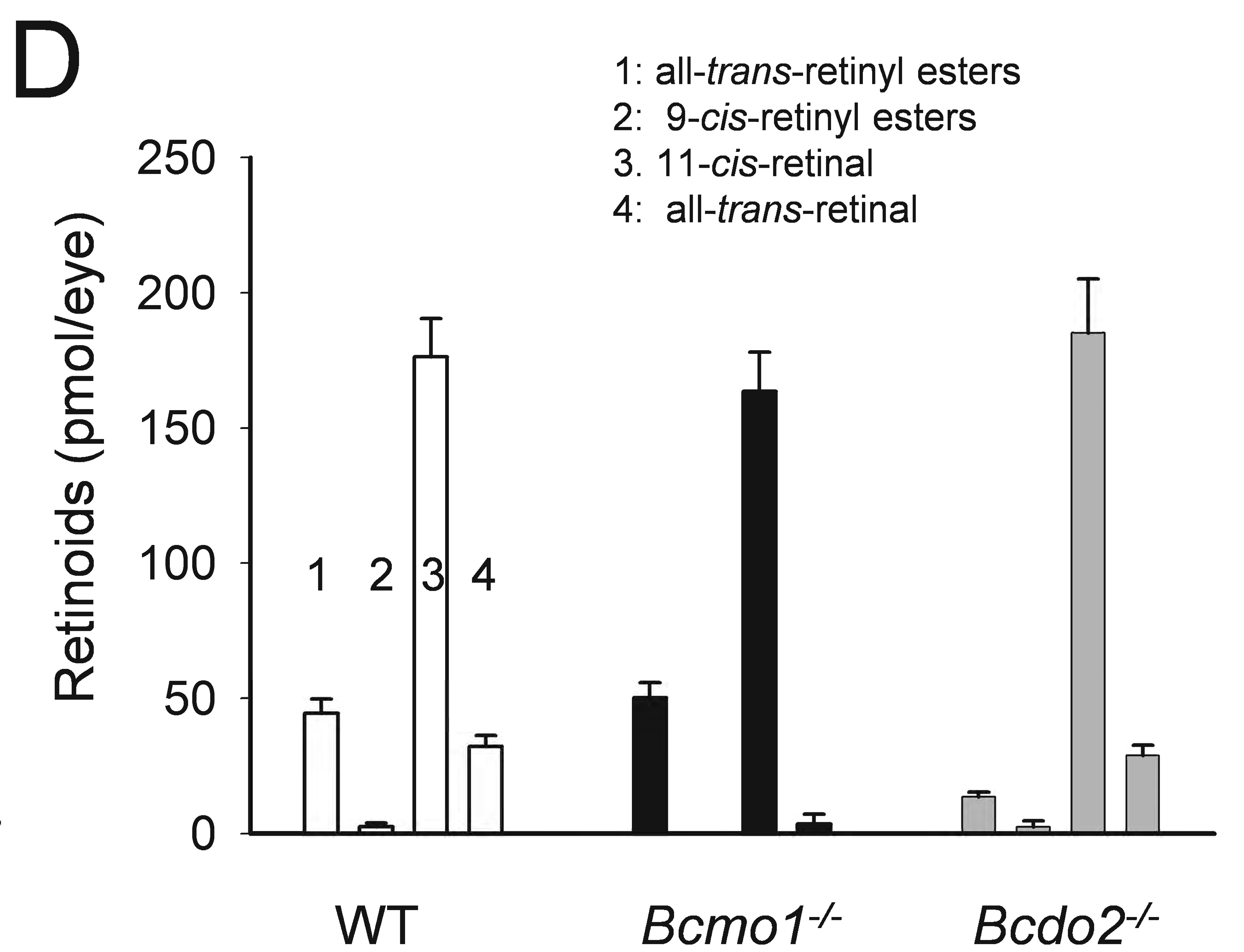
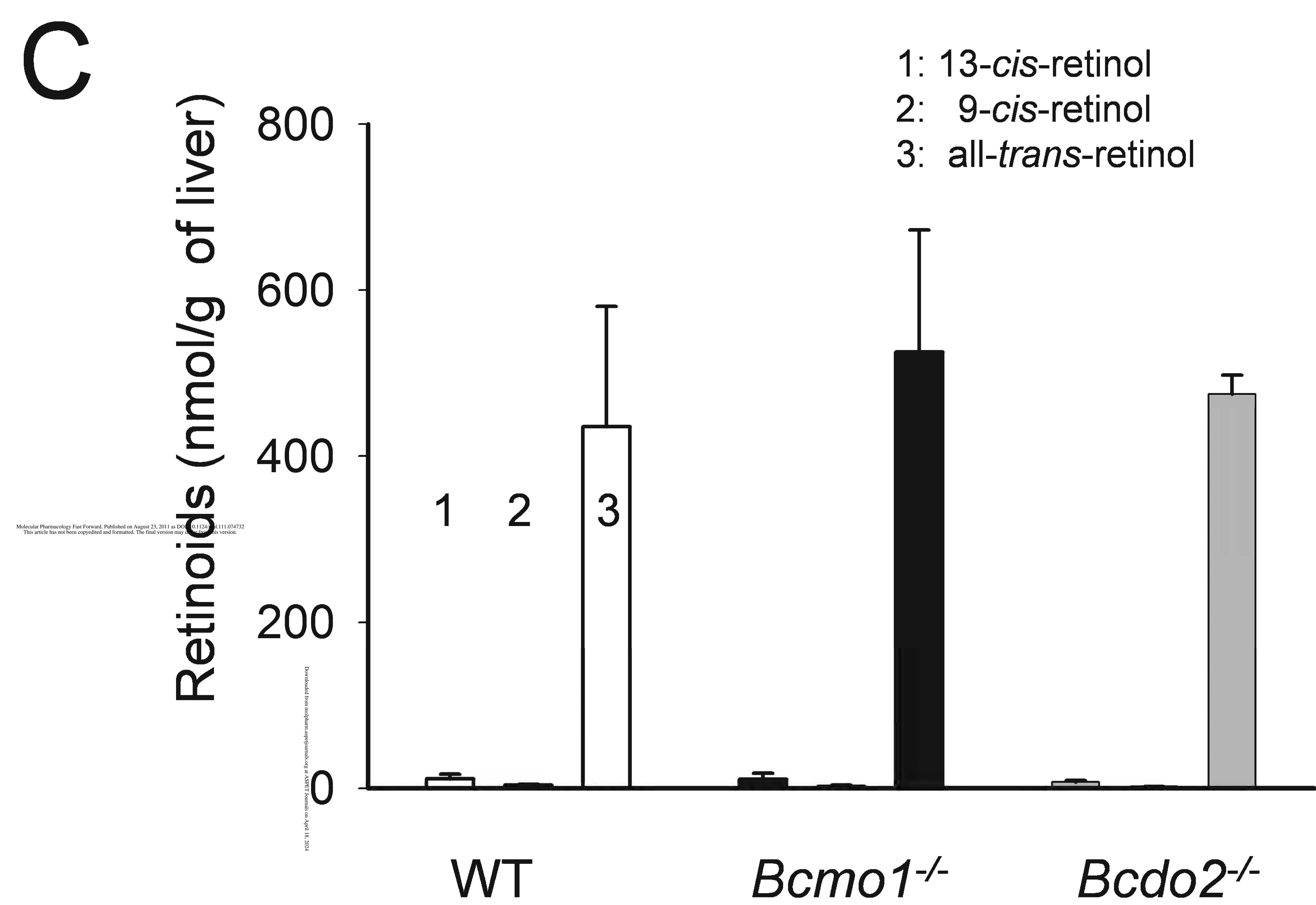
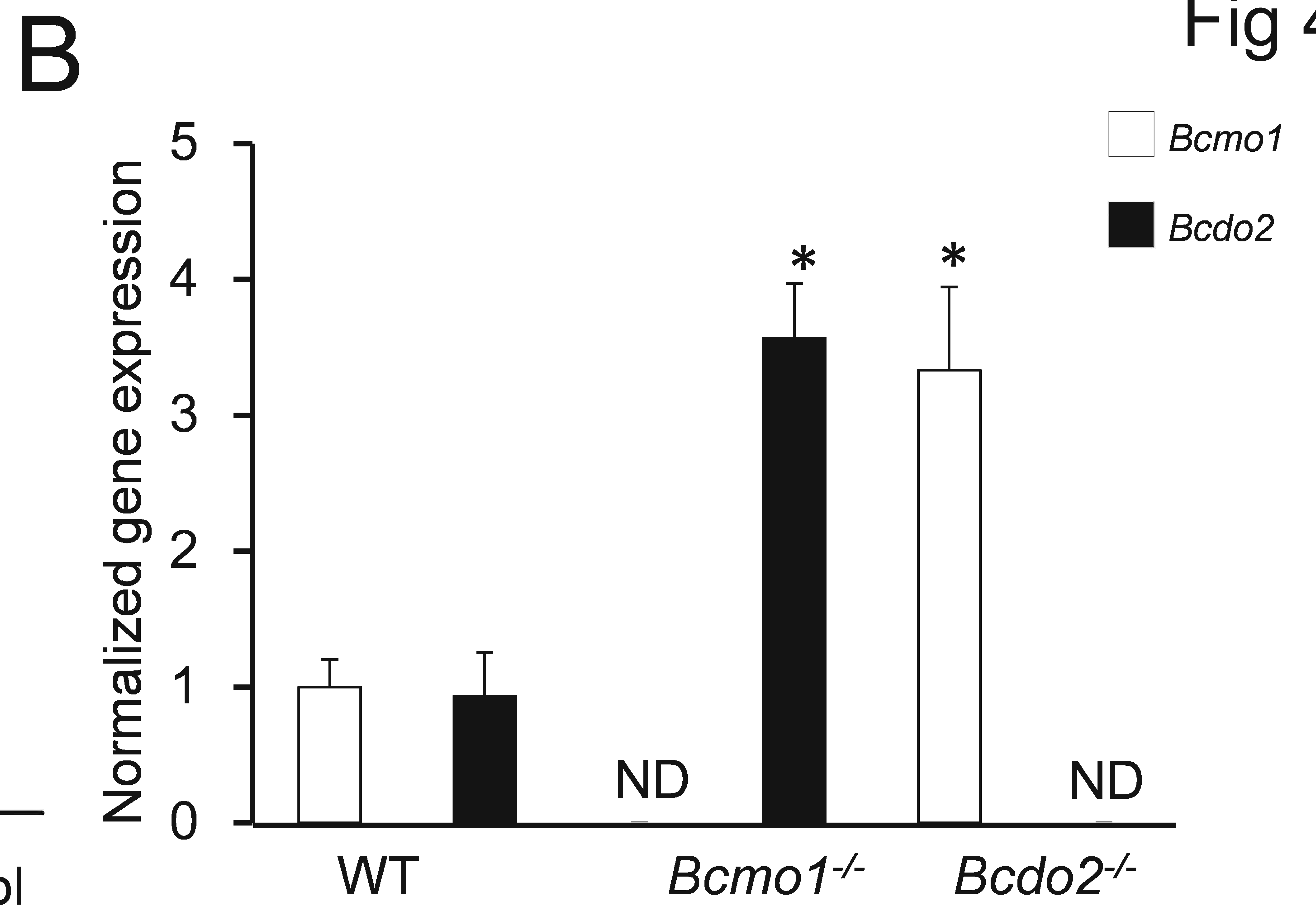
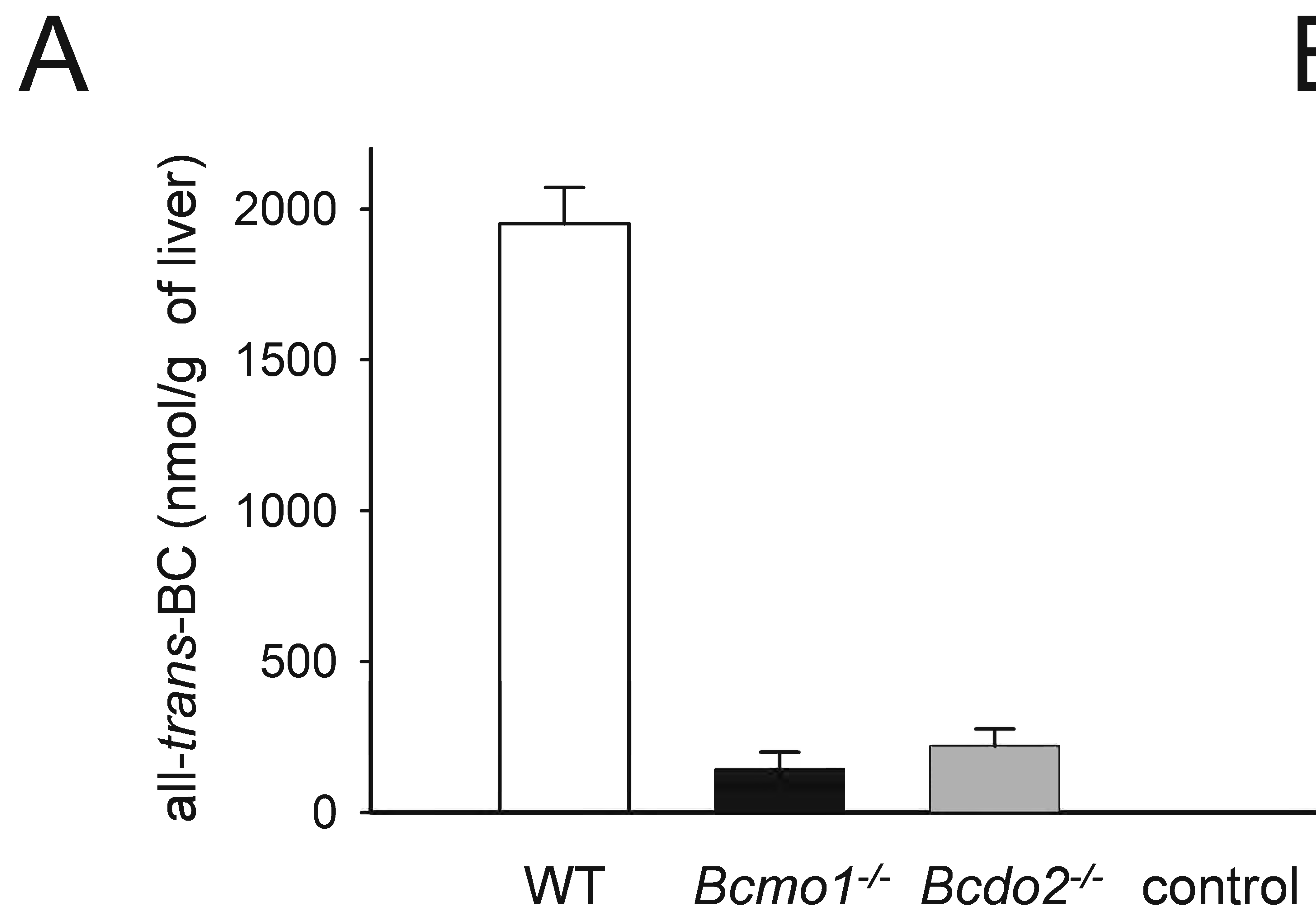


Fig 4



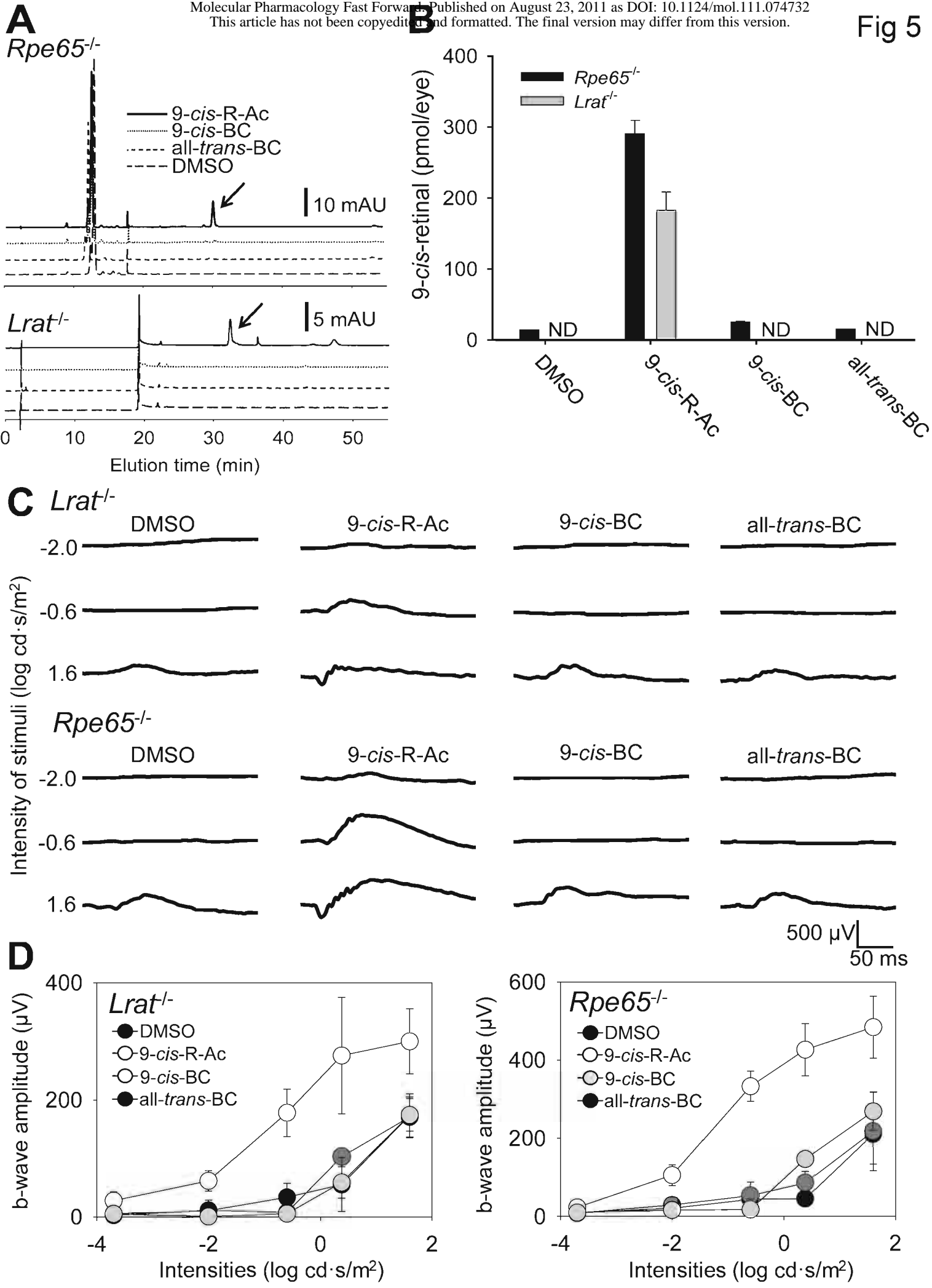


Fig 6

