Cytochrome P450 Oxidase 2C Inhibition Adds to \(\omega-3\) Long-Chain Polyunsaturated Fatty Acids Protection Against Retinal and Choroidal Neovascularization


Objective—Pathological ocular neovascularization is a major cause of blindness. Increased dietary intake of \(\omega-3\) long-chain polyunsaturated fatty acids (LCPUFA) reduces retinal neovascularization and choroidal neovascularization (CNV), but \(\omega-3\) LCPUFA metabolites of a major metabolizing pathway, cytochrome P450 oxidase (CYP) 2C, promote ocular pathological angiogenesis. We hypothesized that inhibition of CYP2C activity will add to the protective effects of \(\omega-3\) LCPUFA on neovascular eye diseases.

Approach and Results—The mouse models of oxygen-induced retinopathy and laser-induced CNV were used to investigate pathological angiogenesis in the retina and choroid, respectively. The plasma levels of \(\omega-3\) LCPUFA metabolites of CYP2C were determined by mass spectroscopy. Aortic ring and choroidal explant sprouting assays were used to investigate the effects of CYP2C inhibition and \(\omega-3\) LCPUFA–derived CYP2C metabolic products on angiogenesis ex vivo. We found that inhibition of CYP2C activity by montelukast added to the protective effects of \(\omega-3\) LCPUFA on retinal neovascularization and CNV by 30% and 20%, respectively. In CYP2C8-overexpressing mice fed a \(\omega-3\) LCPUFA diet, montelukast suppressed retinal neovascularization and CNV by 36% and 39% and reduced the plasma levels of CYP2C8 products. Soluble epoxide hydrolase inhibition, which blocks breakdown and inactivation of CYP2C \(\omega-3\) LCPUFA–derived active metabolites, increased oxygen-induced retinopathy and CNV in vivo. Exposure to selected \(\omega-3\) LCPUFA metabolites of CYP2C significantly reversed the suppression of both angiogenesis ex vivo and endothelial cell functions in vitro by the CYP2C inhibitor montelukast.

Conclusions—Inhibition of CYP2C activity adds to the protective effects of \(\omega-3\) LCPUFA on pathological retinal neovascularization and CNV.

Key Words: arachidonic acid ■ choroidal neovascularization ■ CYP2C inhibitor ■ diabetic retinopathy ■ docosahexaenoic acid}

Pathological ocular angiogenesis comprising retinopathy and choroidal neovascularization (CNV) is a leading cause of vision loss in all age groups, including retinopathy of prematurity in children, diabetic retinopathy, and age-related macular degeneration (AMD) in adults.\(^1\)\(^-\)\(^3\) Pathological retinal neovascularization and CNV can be suppressed temporarily with antiangiogenic agents. In particular, anti–vascular endothelial growth factor (VEGF) molecules have been used successfully for the treatment of neovascular AMD, diabetic retinopathy, and retinopathy of prematurity but with some adverse effects.\(^4\)\(^-\)\(^5\) Suppressing VEGF signaling does not address the underlying causes of neovascularization. Frequent intraocular injections of anti-VEGF drugs also carry a cumulative risk of complications, including the potential of long-term suppression of the beneficial neurotrophic effects of VEGF on neural retina.\(^6\)\(^-\)\(^7\) Identification of additional therapies with fewer adverse effects is highly desirable.

Beyond their roles as energy substrates and structural constituents of membranes, essential dietary lipids and their metabolites also regulate retinal neovascularization and CNV.\(^8\)\(^-\)\(^10\) In particular, long-chain polyunsaturated fatty acids (LCPUFA) influence eye diseases.\(^11\) The \(\omega-3\) LCPUFA, docosahexaenoic acid available at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.307558/-/DC1. The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.307558/-/DC1. Correspondence to Lois E.H. Smith, MD, PhD, Department of Ophthalmology, Boston Children’s Hospital, 300 Longwood Avenue, Boston, MA 02115. Email lois.smith@childrens.harvard.edu

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angiogenesis in retina and choroid in these models.\textsuperscript{8,18} 5-lipoxygenase–derived 4-hydroxy-docosahexaenoic acid, whereas ω-3 LCPUFA are metabolized by at least 3 ω-6 LCPUFA from these pathways, are generally proangiogenic,\textsuperscript{11} whereas ω-3 LCPUFA metabolites of the first 2 pathways, such as cyclooxygenase–2–derived prostaglandin E\textsubscript{3} and 5-lipoxygenase–derived 4-hydroxy-docosahexaenoic acid, show antiangiogenic effects.\textsuperscript{10,19} Our previous studies found that CYP2C products derived from ω-3 and ω-6 LCPUFA, particularly 19,20-epoxydocosapentaenoic acid (EDP) and 14,15-epoxyeicosatrienoic acid (EET), are proangiogenic and involved in CYP2C regulation of retinal neovascularization,\textsuperscript{20} partially counteracting the overall antiangiogenic effects of ω-3 LCPUFA. These bioactive epoxides are further hydrolyzed by soluble epoxide hydrolase (sEH) into less active diols, such as 19,20-dihydroxy-docosapentaenoic acid and 14,15-dihydroxy-eicosatrienoic acid.\textsuperscript{20,21} These findings suggest that inhibition of CYP2C activity might add to the protective effects of ω-3 LCPUFA on pathological retinal neovascularization and CNV.

Both ω-3 and ω-6 LCPUFA are metabolized by at least 3 major pathways: cyclooxygenases, lipoxygenases, and cytochrome P450 oxidases (CYPs). The metabolic products of ω-3 LCPUFA from these pathways are generally proangiogenic,\textsuperscript{11} whereas ω-3 LCPUFA metabolites of the first 2 pathways, such as cyclooxygenase–2–derived prostaglandin E\textsubscript{3} and 5-lipoxygenase–derived 4-hydroxy-docosahexaenoic acid, show antiangiogenic effects.\textsuperscript{10,19} Our previous studies found that CYP2C products derived from ω-3 and ω-6 LCPUFA, particularly 19,20-epoxydocosapentaenoic acid (EDP) and 14,15-epoxyeicosatrienoic acid (EET), are proangiogenic and involved in CYP2C regulation of retinal neovascularization,\textsuperscript{20} partially counteracting the overall antiangiogenic effects of ω-3 LCPUFA. These bioactive epoxides are further hydrolyzed by soluble epoxide hydrolase (sEH) into less active diols, such as 19,20-dihydroxy-docosapentaenoic acid and 14,15-dihydroxy-eicosatrienoic acid.\textsuperscript{20,21} These findings suggest that inhibition of CYP2C activity might add to the protective effects of ω-3 LCPUFA on pathological retinal neovascularization and CNV.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**CYP2C Inhibition Added to the Protective Effects of ω-3 LCPUFA on Retinal Neovascularization and CNV**

To test the hypothesis that inhibiting CYP2C activity adds to ω-3 LCPUFA inhibition of pathological retinal neovascularization and CNV, we subjected C57BL/6 mice fed with either ω-6 or ω-3 LCPUFA–enriched diets (as 2% of total dietary fatty acids are 2% arachidonic acid without any ω-3 LCPUFA or 1% DHA and 1% eicosapentaenoic acid without any ω-6 LCPUFA) to either OIR or laser-induced CNV and treated them with either the CYP2C inhibitor montelukast or vehicle control. At postnatal day (P) 17, OIR pups fed a ω-3 (versus ω-6) LCPUFA–enriched diet had 19% (P=0.046) less retinal neovascularization, which was further reduced by the inhibition of CYP2C by 30% (P=4.4×10\textsuperscript{-7}; Figure 1A and 1B). Moreover, adult mice fed a ω-3 (versus ω-6) LCPUFA–enriched diet had 10% (P=0.020) reduction of CNV lesion area at 7 days after laser photocagulation, and inhibition of CYP2C further reduced the CNV lesion area by 20% (P=6.6×10\textsuperscript{-5}; Figure 1C and 1D). Although the effects of CYP2C inhibition are not specific for, or require ω-3 LCPUFA, these results suggested that CYP2C inhibitor montelukast enhances the overall protective effects of ω-3 LCPUFA on both retinal neovascularization and CNV.

### Inhibition of CYP2C8 Activity Suppressed Retinal Neovascularization and CNV and CYP2C8 Products

To further examine the potential for CYP2C inhibition to augment the protective effect of ω-3 LCPUFA on ocular neovascularization, we treated Tie2-driven human CYP2C8–overexpressing mice and their wild-type littermates on a ω-3 LCPUFA–enriched diet with montelukast or vehicle control daily in the OIR and laser-induced CNV models. CYP2C8 overexpression increased retinal neovascularization in OIR by 34% (P=3.0×10\textsuperscript{-3}), which was suppressed by 36% (P=1.7×10\textsuperscript{-5}) at P17 with montelukast treatment.
CYP2C Inhibitor Reduces Pathological Angiogenesis

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CYP2C8 transgenic mice fed a ω-3 LCPUFA–enriched diet had 25% (P=3.4×10−7) more CNV than wild-type littermates, which was suppressed by 39% (P=3.4×10−16) with montelukast treatment (Figure 2B; Figure IB in the online-only Data Supplement). We chose to examine the plasma levels of arachidonic acid and DHA metabolites of CYP2C, 14,15-EET and 19,20-EDP, and their metabolic products of sEH (Figure 2C) because our previous studies revealed their proangiogenic effects on pathological retinal neovascularization and CNV. The decreased pathological angiogenesis with CYP2C inhibition was accompanied by 24% (P=0.011) lower plasma levels of the bioactive CYP2C8 product derived from ω-3 LCPUFA, 19,20-EDP (Figure 2D). The production of other LCPUFA metabolites of CYP2C8 was also suppressed by montelukast (Table I in the online-only Data Supplement). Also, montelukast reversed the induction of retinal neovascularization (OIR) and CNV in the Tie2-driven CYP2C8 transgenic mice on a ω-6 LCPUFA–enriched diet by 51% (P=8.6×10−4) and 47% (P=6.5×10−7), respectively (Figures I and II in the online-only Data Supplement).

Figure 1. Cytochrome P450 oxidase (CYP) 2C inhibitor added to the protective effects of ω-3 ω-3 long-chain polyunsaturated fatty acids (LCPUFA) on retinal and choroidal neovascularization (NV). Representative images of retinal (A) and choroidal (C) flat-mounts from oxygen-induced retinopathy (OIR) mice fed with a ω-6 or ω-3 LCPUFA–enriched diet and intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% dimethyl sulfoxide control (Ctrl) daily from postnatal day (P) 12 to P16 for OIR or from day 0 to day 6 after laser photocoagulation for choroidal NV. Scale bar=1 mm (A) and 500 µm (C). CYP2C inhibitor augmented the suppression of retinal (B) and choroidal (D) NV by ω-3 LCPUFA. n=11 mice/group. *P<0.05; **P<0.01; ***P<0.001.

Figure 2. Cytochrome P450 oxidase (CYP) 2C inhibitor reduced retinal and choroidal neovascularization (NV) and CYP2C8 products. CYP2C inhibitor reversed the induction of retinal (A) and choroidal (B) NV by CYP2C8 overexpression in the mouse oxygen-induced retinopathy (OIR) and laser-induced choroidal NV models. Tie2-driven CYP2C8 transgenic (Tg) mice and wild-type (WT) littermate controls (Ctrl) fed with a ω-3 long-chain polyunsaturated fatty acid–enriched diet were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% dimethyl sulfoxide control daily from postnatal day (P) 12 to P16 for OIR or from day 0 to day 6 after laser photocoagulation for CNV. C. Schematic diagram of CYP2C8 and soluble epoxide hydroxase (sEH) metabolism of docosahexaenoic acid (DHA) and arachidonic acid (AA). D. CYP2C inhibitor reversed the induction of plasma levels of DHA metabolites downstream of CYP2C8, 19,20-epoxycosapentaenoic acid (EDP), by CYP2C8 overexpression in mice. n=10 to 16 mice/group. *P<0.05; **P<0.01; ***P<0.001. DHET indicates dihydroxy-eicosatrienoic acid; DiHDPA, dihydroxy-docosapentaenoic acid; EDP, epoxydocosapentaenoic acid; and EET, epoxyeicosatrienoic acid.
Data Supplement), which was accompanied by 23% (P=0.031) lower plasma levels of CYP2C8 products derived from ω-6 LCPUFA, such as 14,15-EET, without changing CYP2C8 transcriptional levels (Figure II and Table II in the online-only Data Supplement). These results suggested that inhibition of CYP2C activity by montelukast is associated with suppression of pathological angiogenesis in OIR and laser-induced CNV.

sEH Inhibition Increased Retinal Neovascularization and CNV In Vivo and Angiogenesis Ex Vivo

To investigate the regulatory effects of the CYP2C/sEH pathway and the metabolites on pathological retinal neovascularization and CNV, we treated C57BL/6J mice with the sEH inhibitor in the piperidine series 1770 (1-trifluoromethoxyphenyl1-3-(1-propionylpiperidin-4-yl)urea) or vehicle control in both OIR and laser-induced CNV models. We found that sEH inhibition increased ocular neovascularization by 27% (P=3.3x10^-8) and 26% (P=9.7x10^-6), respectively, in OIR and laser-induced CNV (Figure 3A and 3B; Figure IIIA and IIIB in the online-only Data Supplement), which was also accompanied by 56±17% (P=0.016) and 105% (P=0.032) higher plasma levels of the bioactive epoxides 19,20-EDP and 14,15-EET (Figure 3C and 3D). However, neither sEH nor CYP2C inhibitor affected vaso-obliteration in OIR (Figure IIIC and IIDD in the online-only Data Supplement). The sEH inhibitor 1770 significantly decreased the plasma levels of diols but had no effect on LCPUFA metabolites through other pathways (Table III in the online-only Data Supplement). The ratios of both 19,20-EDP:dihydroxy-docosapentaenoic acid and 14,15-EET:dihydroxy-eicosatetraenoic acid were increased by 153% (P=0.035) and 44% (P=0.020), respectively, by addition of the sEH inhibitor 1770 (Figure IVA and IVB in the online-only Data Supplement). The transcriptional levels of neither

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Soluble epoxide hydrolase (sEH) inhibitor aggravated retinal and choroidal neovascularization. sEH inhibitor increased postnatal day (P) 17 retinal (A) and choroidal (B) neovascularization (NV) in oxygen-induced retinopathy (OIR) and laser-induced choroidal NV mice. C57BL/6J mice were intraperitoneally injected with the sEH inhibitor 1770 (0.3 mg/kg) and 10% dimethyl sulfoxide control daily from P12 to P16 for OIR (n=26–27 mice/group) or from day 0 to day 6 after laser photocoagulation for choroidal NV (n=11 mice/group). sEH inhibitor increased plasma levels of 19,20-epoxydocosapentaenoic acid (EDP; C) and 14,15-epoxyeicosatrienoic acid (EET; D) in mouse. *P<0.05; ***P<0.001.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Soluble epoxide hydrolase (sEH) inhibitor promoted angiogenesis ex vivo. Representative images of aortic rings (A) and choroidal (C) sprouting treated with the sEH inhibitor 1770 (20 µg/mL) or 0.2% dimethyl sulfoxide (DMSO) as control for 6 days after tissue planting. Scale bar=1 mm. sEH inhibitor promoted aortic ring (B) and choroidal (D) sprouting. *P<0.05; ***P<0.001.
CYP2C nor sEH were significantly altered by sEH inhibition (Figure IVC and IVD in the online-only Data Supplement). Moreover, sEH inhibition yielded 48% \((P=5.0\times10^{-5})\) and 42% \((P=0.046)\) increases in sprouting of both aortic rings and choroidal explants (Figure 4). These results indicated the association of \(\omega-3\) LCPUFA metabolites of CYP2C with neovascularization.

**19,20-EDP Reversed the Inhibition of Angiogenesis Ex Vivo by CYP2C Inhibition**

To further investigate whether inhibition of CYP2C and lower levels of CYP2C products derived from \(\omega-3\) LCPUFA suppressed angiogenesis, we examined the effects of the CYP2C inhibitor montelukast in the presence of DHA, which would provide metabolites from many pathways including beneficial products or one of its CYP2C metabolites, 19,20-EDP, on tissue explants. In the aortic ring sprouting assay, there was 19% \((P=0.030)\) less sprouting area with DHA compared with control at day 6 (Figure VA and VB in the online-only Data Supplement). Addition of montelukast further suppressed sprouting by 46% \((P=0.0037)\), which was consistent with results from the animal models in vivo, suggesting that CYP2C products were proangiogenic. In the choroid sprouting assay, montelukast not only decreased the sprouting area by 29% \((P=0.0087)\) but also further increased the inhibitory effects of DHA by 28% \((P=0.013)\;\text{Supplemental Figure VC and VD in the online-only Data Supplement}\). In contrast, one of the DHA metabolites of CYP2C, 19,20-EDP, reversed the suppression of sprouting by montelukast in aortic rings and choroidal explants by 58% \((P=4.3\times10^{-4})\) and 59% \((P=4.2\times10^{-4})\), respectively (Figure 5). These data suggested that inhibition of CYP2C by montelukast suppressed angiogenesis ex vivo.

**19,20-EDP Reversed the Inhibition of Endothelial Cell Tubule Formation In Vitro by CYP2C Inhibition**

We examined the antiangiogenic effects of the CYP2C inhibitor montelukast on endothelial cell tubule formation in vitro using human retinal microvascular endothelial cells (HRMECs). Cells treated with montelukast showed a 32% reduction \((P=3.5\times10^{-4})\) in tubule formation compared with cells treated with control (Figure 6A and 6B). Addition of DHA further inhibited HRMEC tubule formation by 64% \((P=0.0025)\) without any effect on CYP2C8 expression (Figure VI in the online-only Data Supplement). However, 19,20-EDP reversed the suppression of HRMEC tubule formation by montelukast by 43% \((P=7.4\times10^{-4})\;\text{Figure 6A and 6B}\). Neither montelukast nor 19,20-EDP had an effect on the transcription of CYP2C8 in HRMECs (Figure 6C). To further distinguish between CYP2C8 and CYSLTR1 inhibitory effects of montelukast, we treated HRMECs with specific CYP2C8 or CYSLTR1 siRNAs. CYP2C8, but not CYSLTR1, knockdown inhibited HRMEC tubule formation by 64% \((P=0.0025)\;\text{Supplemental Figure VIIC in the online-only Data Supplement}\). Moreover, CYSLTR1 expression levels were undetectable in HRMECs (Figure VIIC in the online-only Data Supplement). These data suggested that the lower levels of bioactive CYP2C products derived from \(\omega-3\) LCPUFA are involved in the impairment of endothelial cell functions by CYP2C inhibition.

### Discussion

Increased dietary intake of \(\omega-3\) LCPUFA reduces pathological retinal neovascularization and CNV.\(^8\) Although many \(\omega-3\) LCPUFA metabolites biosynthesized through cyclooxygenase
and lipoxynase pathways have antiangiogenic effects, ω-3 LCPUFA metabolites of CYP2C promote both retinal neovascularization (OIR) and CNV (laser-induced CNV). In this study, we showed that inhibition of CYP2C activity by montelukast added to the protective effects of ω-3 LCPUFA on ocular neovascularization in the animal models of both OIR and laser-induced CNV. The increased neovascularization observed in transgenic mice overexpressing human CYP2C8 on a ω-3 LCPUFA–enriched diet was substantially reversed with CYP2C inhibition, associated with lower plasma levels of CYP2C8 products derived from ω-3 LCPUFA without affecting CYP2C8 transcriptional levels. Montelukast treatment reduced both CNV and OIR in CYP2C8 transgenic mice to the level of or below that of untreated wild-type litters. In addition, inhibition of sEH significantly increased ocular neovascularization in vivo in association with increased plasma levels of the bioactive ω-3 LCPUFA metabolites of CYP2C2. Addition of ω-3 LCPUFA metabolites of CYP2C reversed the suppression of angiogenesis ex vivo and endothelial cell functions in vitro by CYP2C inhibition.

CYP2C8 is present at high levels in human tissues and is involved in endothelial cell functions. In an in vitro screening of 209 frequently prescribed drugs to examine their potential to inhibit CYP2C8, montelukast was identified as a potent suppressor and a selective inhibitor of CYP2C8 activity among all tested human CYP2C isoforms. Mouse CYP2C55 shares a high homology with human CYP2C8. In our studies, montelukast reduced retinal neovascularization and CNV not only in Tie2-driven CYP2C8 (human) transgenic mice but also in wild-type mice, which suggests that montelukast inhibits not only exogenous human CYP2C8 but also endogenous mouse CYP2C activity, such as CYP2C55.

Montelukast was originally formulated as a specific antagonist for CYSLTR1 and is broadly used to treat chronic asthma. CYSLTR1 is expressed in lung, spleen, leukocytes, macrophages, and smooth muscle cells, and its activation leads to contraction and proliferation of smooth muscle, edema, and eosinophilic migration. Studies of the effect of montelukast on angiogenesis are limited. Montelukast suppresses colon cancer growth through the inhibition of angiogenesis and inhibits angiogenesis ex vivo in rat thoracic aortic rings. Our data show that montelukast inhibited angiogenesis ex vivo in mouse aortic rings and choroid explants. However, there is some controversy about the effects of montelukast on endothelial cells. Montelukast was reported to reduce vascular permeability by reducing VEGF expression, whereas others found that montelukast increases intercellular adhesion molecule 1 expression in human primary endothelial cells. CYSLTR1 is generally reported as absent in primary endothelial cells, although one group reported its expression in a human endothelial cell line and that montelukast inhibited endothelial cell migration by inhibiting the extracellular signal-regulated kinase pathway. We also observed the inhibition of human retinal endothelial cell migration and VEGF-induced extracellular signal-regulated kinase activation by montelukast (Figure VIII in the online-only Data Supplement) but failed to detect the expression of CYSLTR1 in human primary retinal endothelial cells (Figure VIIC in the online-only Data Supplement). Our results suggested that montelukast impairs endothelial cell functions by inhibiting CYP2C activity. The CYP2C inhibitor montelukast is approved by the US Food and Drug Administration and has been used to treat asthma for decades so could be repurposed to treat neovascular eye diseases. Our findings enhance our knowledge of tissue-specific effects of montelukast and its mechanism of angiogenesis regulation.

Compared with ω-6 LCPUFA, ω-3 LCPUFA reduce pathological retinal neovascularization and CNV. The ω-3 LCPUFA DHA inhibits angiogenesis ex vivo and endothelial functions in vitro, which is consistent with previous reports and likely related to its metabolites produced through cyclooxygenase and lipoxynase, rather than CYP pathways. DHA does not appreciably alter the effects of CYP2C inhibition and further increases the inhibition of angiogenesis, suggesting that DHA has parallel effects in the cotreatment with a CYP2C inhibitor. In contrast to the modest effects of DHA on the inhibition of montelukast, addition of CYP2C products derived from DHA, such as 19,20-EDP, reversed the inhibitory effects of this CYP2C inhibitor on angiogenesis ex vivo and endothelial cell functions in vitro, suggesting that montelukast functions upstream of CYP2C.
products. Moreover, increased plasma levels of CYP2C products derived from ω-3 LCPUFA with inhibition of sEH activity also promoted neovascularization, which was accompanied by increased levels of ω-3 LCPUFA metabolites of CYP2C in plasma. Our results suggested that inhibition of CYP2C activity suppresses endothelial cell growth during angiogenesis by lowering the levels of CYP2C products. More clinical research on biological effects of CYP2C inhibition on neovascularization will expand our understanding about the mechanism of CYP2C/sEH pathway regulation of angiogenesis.

CYP2C inhibition reversed the induction of retinal neovascularization and CNV in CYP2C8-overexpressing mice fed with either a ω-3 or a ω-6 LCPUFA–enriched diet. These results suggest that CYP2C products derived from both ω-3 and ω-6 LCPUFA are proangiogenic in the retina and choroid. A previous study showed that 19,20-EDP inhibited tumor growth and human umbilical vein endothelial cell functions in vitro by suppressing VEGF-C but not VEGF-A.39 In our studies, no change in VEGF-C expression was observed in either Tie2-driven CYP2C8 transgenic or CYP2C inhibitor–treated retina (Figure IX in the online-only Data Supplement). The different expression pattern of VEGF-A and VEGF-C in the retina and in tumor might contribute to the different effects of the CYP2C metabolite observed, indicating a tissue-specific role of 19,20-EDP. Despite much research on the potent effects of other CYP2C metabolites, such as EDPs and EETs, in many physiological and pathological processes, knowledge about the molecular mechanism or regulation of angiogenesis and endothelial cell behaviors is still limited. A recent study indicated that 11,12-EET promotes hematopoietic stem and progenitor cell specification by increasing activator protein 1 and runx1 transcription through phosphatidylinositol-3-OH kinase pathway,40 which has also been implicated in advanced AMD by our previous work.41 More research on the direct target and downstream pathways of CYP2C metabolites is needed.

Our inferences were strengthened on the basis of 5 additional lines of evidence: (1) intravitreal injections of trinethoprin, an antibiotic known to inhibit CYP2C8, is effective in treatment of human toxoplasma retinochoroiditis, a disease affecting the retinal vasculature42,43; (2) injections of cationic liposome-encapsulated paclitaxel, a substrate of CYP2C8, reduces the volume of CNV in an animal model of pathological choroidal angiogenesis44; (3) exposure to thiazolidinedione, an antidiabetic agent known to inhibit CYP2C8, significantly reduces the extent of retinal neovascular pathology in OIR mice45—effects in this report were attributed to a pioglitazone-induced increase in adiponectin, a ω-3 LCPUFA–modulated protective factor for pathological retinal neovascularization and CNV46; (4) CYP2C8–inhibiting thiazolidinediones also act as trans activators of the peroxisome proliferator activated receptor gamma transcription response element, which is part of an ω-3 LCPUFA–signaling system implicated in retinopathy of prematurity, diabetic retinopathy, and AMD47,48; and (5) pull-down and coimmunoprecipitation studies confirmed the binding of CYP2C8 with alpha 2-macroglobulin, a major protease inhibitor that acts on and is cleaved by matrix metalloproteinase 9 an AMD-associated collagenase essential for clearing space for sprouting vessels within the angiogenic cascade.49,50

In summary, our study found that inhibition of CYP2C inhibited pathological retinal neovascularization and CNV by lowering the levels of CYP2C products from both ω-6 and ω-3 LCPUFA. ω-3 LCPUFA may help prevent retinal neovascularization and CNV. Our findings suggest enhanced protective effects of ω-3 LCPUFA against pathological angiogenesis with CYP2C inhibition. Montelukast is a potential therapeutic to treat neovascular eye diseases. Dietary ω-3 LCPUFA DHA supplementation with CYP2C inhibition is likely to benefit retinal neovascularization and CNV.

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Disclosures

None.

References

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**Highlights**

- Cytochrome P450 oxidase 2C inhibitor reduces retinal and choroidal neovascularization.
- Soluble epoxide hydrolase inhibitor induces retinal and choroidal neovascularization.
- Cytochrome P450 oxidase 2C inhibitor adds to the protective effects of ω-3 long-chain polyunsaturated fatty acids on pathological retinal and choroidal neovascularization.
- Cytochrome P450 oxidase inhibitor presents a new therapeutic approach for prevention of neovascular eye diseases.
- Montelukast as an approved pharmaceutical on the market could be repurposed for treating neovascular eye diseases.
Cytochrome P450 Oxidase 2C Inhibition Adds to ω-3 Long-Chain Polyunsaturated Fatty Acids Protection Against Retinal and Choroidal Neovascularization

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Materials and Methods

Mice
Tie2-driven CYP2C8 overexpressing transgenic mice were in the C57BL/6N genetic background\(^1\), and wild-type C57BL/6J mice were purchased from the Jackson Laboratory (000664, Bar Harbor, ME). For dietary experiments, ω-6 long-chain polyunsaturated fatty acids (LCPUFA), arachidonic acid (AA), and ω-3 LCPUFA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were obtained from DSM Nutritional Products (TE Heerlen, Netherlands) and integrated into the rodent feed at Research Diets (New Brunswick, NJ)\(^2\, 3\). The feed (TS00002988, DSM) was analyzed to confirm composition and there were no peroxides, no dioxin, no benzopyrene or any heavy metal contaminants (EPA1T1615.07/111001). The mice were fed the defined rodent diet with 10% (w/w) safflower oil containing either 2% ω-6 LCPUFA (AA) and no ω-3 LCPUFA (DHA and EPA), or 2% ω-3 LCPUFA and no ω-6 LCPUFA from postnatal day (P) 1 to P17 for the mouse model of oxygen-induced retinopathy (OIR) or from 7 days before laser photocoagulation for the mouse model of laser-induced choroidal neovascularization. All studies adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Boston Children's Hospital Animal Care and Use Committee.

Oxygen-induced retinopathy
The mouse OIR model\(^4\, 5\) was used to induce pathological retinal neovascularization. Mouse pups and their nursing mother were exposed to 75±3 % oxygen from P7 to P12. The cytochrome P450 oxidase (CYP) 2C inhibitor montelukast sodium was purchased from Santa Cruz Biotechnology (sc-202231, Dallas, TX), and dissolved in dimethyl sulfoxide (DMSO, D2650, Sigma-Aldrich, St. Louis, MO) to make a 10 mg/ml stock solution. The sEH inhibitor 1770 was dissolved in DMSO to make a 3 mg/ml stock solution. After being returned to room air, mice were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg), the sEH inhibitor 1770 (0.3 mg/kg) or 10% DMSO as control daily from P12 to P16. At P17, eyes were immediately enucleated after euthanasia and fixed in 4% paraformaldehyde (PFA, P6148, Sigma-Aldrich) in phosphate buffered saline (PBS, 10010-023, Thermo Fisher Scientific, Waltham, MA) for 1 hour at room temperature. Retinas were then dissected and stained overnight with Alexa Fluor 594 conjugated isolectin GS-IB\(_4\) (10 μg/ml, I21413, Thermo Fisher Scientific) at room temperature. After being washed with PBS, retinas were whole-mounted onto microscope slides (12-550-15, Thermo Fisher Scientific) with photoreceptor side down and embedded in SlowFade antifade mounting medium (S2828, Thermo Fisher Scientific). Retinal images were taken by a fluorescence microscope (AxioObserver.Z1, Carl Zeiss Microscopy, Jena, Germany) with image software (AxioVision 4.6.3.0, Carl Zeiss Microscopy). Retinal neovascularization was analyzed with the SWIFT_NV method on ImageJ (National Institute of Health)\(^6\).

Laser-induced choroidal neovascularization
The mouse model of laser-induced choroidal neovascularization was used as reported\(^7\). Laser photocoagulation was induced using an image-guided laser system (Micron IV, Phoenix Research Laboratories, Pleasanton, CA) in mice at the age of 6-8 weeks. Four laser burns at equal distance from the optic nerve head were generated in each eye by a green Argon laser pulse with a wavelength of 532 nm, a fixed diameter of 50 μm, duration of 70 ms, and power level of 240 mW. Eyes were enucleated 7 days after laser photocoagulation and fixed with 4% PFA in PBS for 1 hour at room temperature. The posterior eye cups consisting of the retinal pigment epithelium/choroid/sclera were dissected and permeabilized with 0.1% Triton X-100 (X100, Thermo Fisher Scientific) in PBS for 1 hour at room temperature. The CNV lesions were stained overnight with isolectin GS-IB\(_4\) (10 μg/ml) at room temperature. After being washed with
PBS, the posterior eye cups were whole-mounted with the scleral side down. Fluorescent images were then taken and the areas of lesions were quantified with ImageJ in a masked fashion.

**LC/MS/MS oxylipid analysis**

Levels of CYP2C and sEH products from both ω-3 and ω-6 LCPUFA in plasma were determined by liquid chromatography, tandem mass spectroscopy (LC/MS/MS) after liquid/liquid extraction with ethyl acetate. Analyses were performed as previously described\textsuperscript{1,3}. Internal standard was added and lipids were extracted, dried and reconstituted exactly for plasma. Online liquid chromatography of extracted samples was performed with an Agilent 1200 Series capillary HPLC (Agilent Technologies, Santa Clara, CA). Separations were achieved using a Phenomenex Luna C18(2) column (5 m, 150X1 mm; Phenomenex, Torrance, CA). Analyses were performed on an MDS Sciex API 3000 equipped with a TurbolonSpray source (Applied Biosystems, Foster City, CA).

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**Choroidal sprouting assay**

Retinal pigment epithelium/choroid/sclera complex (also referred to as “choroid explant”) from 3 weeks old C57BL/6J mice was dissected and cut into approximately 1 mm x 1 mm pieces. The choroid explants were then embedded into growth factor reduced Matrigel, cultured and imaged with similar methods as those used for the aortic ring assay described above\textsuperscript{9}.

**Tubule formation assay**

Human retinal microvascular endothelial cells (HRMECs, ACBRI 181, Cell systems) were cultured in endothelial cell growth medium (EGM-2 MV, cc-3202, Lonza, Basel, Switzerland) and used from passage 5 to 8. Cells were seeded onto plates pre-coated with growth factor reduced Matrigel at a density of 2\times10^5 cells/ml, and incubated in EGM-2 MV supplemented with montelukast (20 μg/ml), 19,20-EDP (1 μM), DHA (30 μM), CYP2C8 Stealth siRNAs (10nM, HSS141764, Thermo Fisher Scientific), CysLTR1 Stealth siRNAs (10nM, HSS116670, Thermo Fisher Scientific) or corresponding vehicle controls at 37°C with 5% CO\textsubscript{2} for 6 hours. Phase contrast photos were taken, and the tubule formation was analyzed with Angiogenesis Analyzer for ImageJ.

**Wound healing assay**

HRMEC were grown to confluence on plates pre-coated with gelatin (0.5 μg/ml, G9391, Sigma-Aldrich). After treatment with mitomycin C (10 μg/ml, Sigma-Aldrich) for 20 min at 37°C, monolayers were washed, scratched with a pipet tip and incubated in EGM-2 MV at 37°C for 24 hours. Phase contrast photos were taken, and cell migration was quantitated by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) at 5 distinct positions\textsuperscript{10}, and all assays were performed in triplicates.
Immunoblot analysis
Immunoprecipitation and immunoblot analysis was performed and quantified as described\textsuperscript{11}. Briefly, for immunoblotting, equal volume of proteins were loaded into SDS–polyacrylamide gels, separated by electrophoresis, and transferred onto nitrocellulose membranes (IB301002, Thermo Fisher Scientific). Antibodies against phosphorylated ERK1/2 (T202/Y204, 20611) and ERK1/2 (137F5) were purchased from Cell Signaling (Danvers, MA).

Qualitative real-time polymerase chain reaction
Total RNA was extracted from retinas or HRMECs using RNeasy Kit (74106, Qiagen, Hilden, Germany), and reverse transcribed using random hexamers and SuperScript III Reverse Transcriptase (Thermo Fisher) according to the manufacturers' instructions\textsuperscript{12, 13}. Quantitative analysis of gene expression was determined using an ABI Prism 7700 Sequence Detection System (Applied BioSystems) and the SYBR Green Master Mix kit (KK4600, Kapa Biosystems, Wilmington, MA) with primers listed below: CYP2C8 (F: 5’-TGTGGTCCTGCTGTCGG, R: 5’-ATATTGGGAATTCGTCTCTT), Cyp2C55 (F: 5’-AATGATCTGGGGGTATTTTCAG, R: 5’-GCGATCCTCGATGCTCCTC), sEH (F: 5’-ATCTGAAGCCAGCCGCGTAC, R: 5’-CTGGGCGAGCAGCAGATTCT), Vegfa (F: 5’-GGAGATCCTTCGAGGAGCACTT, R: 5’-GGCGATTTAGCAGCAGATATAAGAA), Vegfc (F: 5’-GAGGTCAAGGCTTTTGAAGGC, R: 5’-CTGTCCTGTATTTGAGGGTGG) and Cyclophilin A (F: 5’-AGGTGGAGAGCACAAAGAGA, R: 5’-TGCCGGAGTCGACAAATGAT). Each target gene cDNA copy number was normalized to the house keeping gene Cyclophilin A using comparative CT (ΔΔCT) method and related to control group.

Statistical analysis
Data are presented as mean ± SEM. Student’s \( t \) test was used to compare between 2 groups of samples. For more than 2 groups of samples, two-way ANOVA with Tukey test was performed using Prism 6 (Graph-Pad, San Diego, CA). \( P < 0.05 \) was considered as statistically significant.

References


Supplemental Figure I. Montelukast reduced ocular neovascularization in mice fed with a ω-3 or ω-6 LCPUFA enriched diet. Representative images of retinal (A & C) or choroidal (B & D) whole-mounts from OIR or laser-induced CNV mice fed with a ω-3 (A & B) or ω-6 (C & D) LCPUFA enriched diet and intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from postnatal day (P) 12 to P16 for OIR mice (A & C) or from day 0 to day 6 after laser photocoagulation for CNV mice (B & D). White arrows indicate pathological angiogenesis in retinal whole-mounts. Scale bar, 1 mm (A & C), 500 μm (B & D).
Supplemental Figure II. Montelukast suppressed ocular neovascularization by inhibiting CYP2C8 activity. A. Montelukast reversed the induction of retinal neovascularization (NV) by CYP2C8 overexpression. Tie2-driven CYP2C8 overexpressing mice and WT littermate controls fed with a ω-6 LCPUFA enriched diet were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from P12 to P16. n = 11-13 mice/group. B. Montelukast reverses the induction of plasma levels of an AA metabolite of CYP2C8, 14,15-EET, by CYP2C8 overexpression in P17 OIR mice. n = 11-13 mice/group. C. Montelukast reduced the laser-induced CNV lesion area induced by CYP2C8 overexpression. Tie2-driven CYP2C8 overexpressing mice and WT littermate controls fed with a ω-6 LCPUFA enriched diet were intraperitoneally injected with montelukast (1 mg/kg) or 10% DMSO control daily from day 0 to day 6 after laser photocoagulation. n = 10 mice/group. D. Montelukast had no effect on CYP2C8 expression in P17 OIR retina. n = 6 mice/group. * P < 0.05; ** P < 0.01; *** P < 0.001; n. s., not significant.
Supplemental Figure III. sEH inhibitor 1770 increased ocular neovascularization.

Representative images of retinal (A) or choroidal (B) whole-mounts from OIR or laser-induced CNV mice intraperitoneally injected with the sEH inhibitor 1770 (0.3 mg/kg) and 10% DMSO as control daily from postnatal day (P) 12 to P16 for OIR mice (A) or from day 0 to day 6 after laser photocoagulation for CNV mice (B). White arrows indicate pathological angiogenesis in retinal whole-mounts. Scale bar, 1 mm (A), 500 µm (B). Neither the sEH inhibitor 1770 (C) nor the CYP2C inhibitor montelukast (D) had a significant effect on vaso-obliteration (VO). n = 20-30 mice/group. n. s., not significant.
Supplemental Figure IV. sEH inhibitor had no effect on the transcriptional levels of CYP2C or sEH. The sEH inhibitor 1770 significantly increased the ratio of 19,20-EDP:DiHDPA (A) and 14,15-EET:DHET (B) in plasma, but had no effect on CYP2C (C) or sEH (D) transcriptional levels in retina. n = 4 mice/group. * P < 0.05; n. s. not significant.
Supplemental Figure V. DHA did not reverse the inhibition of angiogenesis ex vivo by CYP2C inhibition. Representative images of aortic rings (A) and choroidal (C) sprouting treated with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and DHA (30 µM) or BSA control for 6 days after tissue planting. DHA did not reverse the inhibition of aortic ring (B) or choroidal (D) sprouting by CYP2C inhibition. Scale bar, 1 mm. n = 5. * P < 0.05; ** P < 0.01; *** P < 0.001.
Supplemental Figure VI. DHA did not reverse the inhibition of human retinal endothelial cell (HRMEC) tubule formation by CYP2C inhibition. A, Representative photos of HRMECs grown on Matrigel for 6 hour with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and DHA (30 µM) or BSA control. Scale bar, 500 µm. B, DHA did not reverse the inhibition of endothelial cell tubule formation by CYP2C inhibition. C, Montelukast had no significant effect on CYP2C8 mRNA levels in HRMECs. n = 6. * P < 0.05; ** P < 0.01; n. s., not significant.
Supplemental Figure VII. 19,20-EDP reversed the inhibition of HRMEC migration by CYP2C inhibition. A, Representative photos of HRMECs 24 hour after a scratch treated with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and 19,20-EDP (1 µM) or ETOH vehicle control. Scale bar, 500 µm. B, 19,20-EDP rescued the inhibition of cell migration by CYP2C inhibition. n = 5. *** P < 0.001. C, Montelukast impaired VEGF-induced ERK phosphorylation in HRMECs.
Supplemental Figure VIII. CYP2C8, but not CysTLR1, deficiency impaired HRMEC tubule formation. A, Representative photos of HRMECs grown on Matrigel for treated for 6 hours with CYP2C or CysTLR1 siRNAs (10nM). Scale bar, 500 µm. B, CYP2C8, but not CysTLR1, siRNAs inhibited HRMEC tubule formation. C, CYP2C8, but not CysTLR1, siRNAs decreased CYP2C8 mRNA levels. CysTLR1 mRNA levels were undetectable in HRMECs. n = 6. *** P < 0.001; n. s., not significant.
Supplemental Figure IX. Montelukast lowered Vegfa but not Vegfc mRNA levels in mouse retina and choroid. Tie2-driven CYP2C8 transgenic (Tg) mice and wild-type (WT) littermate controls fed with a ω-3 LCPUFA enriched diet were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from P12 to P16 for OIR mice (A & B) or from day 0 to day 6 after laser photocoagulation for CNV mice (C & D). Montelukast reversed the induction of mRNA levels of Vegfa (A & C) but had no effect on Vegfc (B & D) expression in P17 OIR retina (A & B) or CNV choroid (C & D) 7 days after laser photocoagulation. n = 6 eyes/group. * P < 0.05; n. s., not significant.
Supplemental Table I. Plasma levels (pg/ml) of other LCPUFA metabolites in WT and CYP2C8 Tg mice fed with a ω-3 LCPUFA enriched diet and treated with the CYP2C8 inhibitor montelukast or DMSO as control.

<table>
<thead>
<tr>
<th>Metabolites measured</th>
<th>WT DMSO</th>
<th>CYP2C8 Tg DMSO</th>
<th>CYP2C8 Tg Montelukast</th>
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</thead>
<tbody>
<tr>
<td>19,20-DiHDPA</td>
<td>51770.33</td>
<td>67596.17*</td>
<td>37378.02†</td>
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<tr>
<td>17,18-DHEQ</td>
<td>108423.0</td>
<td>165447.7*</td>
<td>91795.67†</td>
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<td>14,15-DHET</td>
<td>3505.881</td>
<td>7209.988*</td>
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<tr>
<td>17,18-EEQ</td>
<td>171.0216</td>
<td>304.7631*</td>
<td>177.0491†</td>
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<tr>
<td>14,15-EET</td>
<td>84.6607</td>
<td>176.3169*</td>
<td>86.44217†</td>
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<tr>
<td>5-HETE</td>
<td>3297.219</td>
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<td>20-HETE</td>
<td>3580.750</td>
<td>5422.105*</td>
<td>4838.885†</td>
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<tr>
<td>PGE2</td>
<td>460.6699</td>
<td>366.2372*</td>
<td>497.6818†</td>
</tr>
<tr>
<td>TXB2</td>
<td>2370.684</td>
<td>1819.801*</td>
<td>2549.474†</td>
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</tbody>
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* significant difference vs. WT DMSO group
† significant difference vs. CYP2C8 Tg DMSO group
DHEQ, dihydroxy-eicosatetraenoic acid
EEQ, epoxyeicosatetraenoic acid
HETE, hydroxyeicosatetraenoic acid
PGE2, prostaglandin E2
TXB2, thromboxane B2
Supplemental Table II. Plasma levels (pg/ml) of other LCPUFA metabolites in WT and CYP2C8 Tg mice fed with a ω-6 LCPUFA enriched diet and treated with the CYP2C8 inhibitor montelukast or DMSO as control.

<table>
<thead>
<tr>
<th>Metabolites measured</th>
<th>WT DMSO</th>
<th>CYP2C8 Tg DMSO</th>
<th>CYP2C8 Tg Montelukast</th>
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<tr>
<td>19,20-DiHDPA</td>
<td>16300.41</td>
<td>29778.95*</td>
<td>16803.31†</td>
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<td>17,18-DHEQ</td>
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<td>14,15-DHET</td>
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<td>20990.71*</td>
<td>9769.505†</td>
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<td>19,20-EDP</td>
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<td>TXB2</td>
<td>4842.415</td>
<td>3054.973*</td>
<td>4567.439†</td>
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* significant difference vs. WT DMSO group
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Supplemental Table III. Plasma levels of LCPUFA metabolites in WT C57BL/6 mice fed with normal feed and treated with the sEH inhibitor 1770 or DMSO as control.

<table>
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<tr>
<th>Metabolites measured</th>
<th>DMSO</th>
<th>sEH inhibitor 1770</th>
</tr>
</thead>
<tbody>
<tr>
<td>19,20-DiHDPA</td>
<td>25735.69</td>
<td>10327.08*</td>
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<td>17,18-DHEQ</td>
<td>40534.88</td>
<td>15806.25*</td>
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<tr>
<td>14,15-DHET</td>
<td>3079.047</td>
<td>1248.125*</td>
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<tr>
<td>19,20-EDP</td>
<td>21538.62</td>
<td>30387.99*</td>
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<td>17,18-EEQ</td>
<td>234.1935</td>
<td>442.7455*</td>
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<td>14,15-EET</td>
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<td>5-HETE</td>
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<tr>
<td>20-HETE</td>
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<tr>
<td>PGE2</td>
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<td>772.9363</td>
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<tr>
<td>TXB2</td>
<td>6709.583</td>
<td>8323.514</td>
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</table>

* significant difference vs. DMSO group
Retinal Neovascularization in Diabetic Retinopathy and Retinopathy of Prematurity

Monelukast

AA → EET

CYP2C

DHA → EDP

Choroidal Neovascularization in Age-related Macular Degeneration
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**Choroidal sprouting assay**
Retinal pigment epithelium/choroid/sclera complex (also referred to as “choroid explant”) from 3 weeks old C57BL/6J mice was dissected and cut into approximately 1 mm ×1 mm pieces. The choroid explants were then embedded into growth factor reduced Matrigel, cultured and imaged with similar methods as those used for the aortic ring assay described above.

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HRMEC were grown to confluence on plates pre-coated with gelatin (0.5 µg/ml, G9391, Sigma-Aldrich). After treatment with mitomycin C (10 µg/ml, Sigma-Aldrich) for 20 min at 37°C, monolayers were washed, scratched with a pipet tip and incubated in EGM-2 MV at 37°C for 24 hours. Phase contrast photos were taken, and cell migration was quantitated by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) at 5 distinct positions, and all assays were performed in triplicates.
Immunoblot analysis
Immunoprecipitation and immunoblot analysis was performed and quantified as described. Briefly, for immunoblotting, equal volume of proteins were loaded into SDS–polyacrylamide gels, separated by electrophoresis, and transferred onto nitrocellulose membranes (IB301002, Thermo Fisher Scientific). Antibodies against phosphorylated ERK1/2 (T202/Y204, 20611) and ERK1/2 (137F5) were purchased from Cell Signaling (Danvers, MA).

Qualitative real-time polymerase chain reaction
Total RNA was extracted from retinas or HRMECs using RNeasy Kit (74106, Qiagen, Hilden, Germany), and reverse transcribed using random hexamers and SuperScript III Reverse Transcriptase (Thermo Fisher) according to the manufacturers’ instructions. Quantitative analysis of gene expression was determined using an ABI Prism 7700 Sequence Detection System (Applied BioSystems) and the SYBR Green Master Mix kit (KK4600, Kapa Biosystems, Wilmington, MA) with primers listed below: CYP2C8 (F: 5'-TGTGGTCTCTGGTGCTGTG, R: 5' ATATTGGGGAATTCCTCTTT), Cyp2C55 (F: 5'-AATGATCTGGGGGTATTTTCAG, R: 5'-GGCGATCTCCAGATGGCTCTC), sEH (F: 5'-ATCTGAAGCCAGCCCGTAC, R: 5'-GCGATCGACAGGAGGATCT), Vegfa (F: 5'-GGAGATCCTTCGAGGAGCACTT, R: 5'-GGCGATTTAGGAGGATAGA), Vegfc (F: 5'-GAGGTCAAGGCTTTTGAAGGC, R: 5'-CTGTCCTGGTATTGAGGGTGG) and Cyclophilin A (F: 5'-AGGTGGAGAGCACCAAGACAGA, R: 5'-TGCCGGAGTCTGCAATGAT). Each target gene cDNA copy number was normalized to the housekeeping gene Cyclophilin A using comparative CT (ΔΔCT) method and related to control group.

Statistical analysis
Data are presented as mean ± SEM. Student’s t test was used to compare between 2 groups of samples. For more than 2 groups of samples, two-way ANOVA with Tukey test was performed using Prism 6 (Graph-Pad, San Diego, CA). P < 0.05 was considered as statistically significant.

References


Supplemental Figure I. Montelukast reduced ocular neovascularization in mice fed with a ω-3 or ω-6 LCPUFA enriched diet. Representative images of retinal (A & C) or choroidal (B & D) whole-mounts from OIR or laser-induced CNV mice fed with a ω-3 (A & B) or ω-6 (C & D) LCPUFA enriched diet and intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from postnatal day (P) 12 to P16 for OIR mice (A & C) or from day 0 to day 6 after laser photocoagulation for CNV mice (B & D). White arrows indicate pathological angiogenesis in retinal whole-mounts. Scale bar, 1 mm (A & C), 500 µm (B & D).
Supplemental Figure II. Montelukast suppressed ocular neovascularization by inhibiting CYP2C8 activity. A. Montelukast reversed the induction of retinal neovascularization (NV) by CYP2C8 overexpression. Tie2-driven CYP2C8 overexpressing mice and WT littermate controls fed with a ω-6 LCPUFA enriched diet were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from P12 to P16. n = 11-13 mice/group. B. Montelukast reverses the induction of plasma levels of an AA metabolite of CYP2C8, 14,15-EET, by CYP2C8 overexpression in P17 OIR mice. n = 11-13 mice/group. C. Montelukast reduced the laser-induced CNV lesion area induced by CYP2C8 overexpression. Tie2-driven CYP2C8 overexpressing mice and WT littermate controls fed with a ω-6 LCPUFA enriched diet were intraperitoneally injected with montelukast (1 mg/kg) or 10% DMSO control daily from day 0 to day 6 after laser photocoagulation. n = 10 mice/group. D. Montelukast had no effect on CYP2C8 expression in P17 OIR retina. n = 6 mice/group. * P < 0.05; ** P < 0.01; *** P < 0.001; n. s., not significant.
Supplemental Figure III. sEH inhibitor 1770 increased ocular neovascularization.
Representative images of retinal (A) or choroidal (B) whole-mounts from OIR or laser-induced CNV mice intraperitoneally injected with the sEH inhibitor 1770 (0.3 mg/kg) and 10% DMSO as control daily from postnatal day (P) 12 to P16 for OIR mice (A) or from day 0 to day 6 after laser photocoagulation for CNV mice (B). White arrows indicate pathological angiogenesis in retinal whole-mounts. Scale bar, 1 mm (A), 500 µm (B). Neither the sEH inhibitor 1770 (C) nor the CYP2C inhibitor montelukast (D) had a significant effect on vaso-obliteration (VO). n = 20-30 mice/group. n. s., not significant.
Supplemental Figure IV. sEH inhibitor had no effect on the transcriptional levels of CYP2C or sEH. The sEH inhibitor 1770 significantly increased the ratio of 19,20-EDP:DiHDPA (A) and 14,15-EET:DHET (B) in plasma, but had no effect on CYP2C (C) or sEH (D) transcriptional levels in retina. n = 4 mice/group. * P < 0.05; n. s. not significant.
Supplemental Figure V. DHA did not reverse the inhibition of angiogenesis ex vivo by CYP2C inhibition. Representative images of aortic rings (A) and choroidal (C) sprouting treated with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and DHA (30 µM) or BSA control for 6 days after tissue planting. DHA did not reverse the inhibition of aortic ring (B) or choroidal (D) sprouting by CYP2C inhibition. Scale bar, 1 mm. n = 5. * P < 0.05; ** P < 0.01; *** P < 0.001.
Supplemental Figure VI. DHA did not reverse the inhibition of human retinal endothelial cell (HRMEC) tubule formation by CYP2C inhibition. A, Representative photos of HRMECs grown on Matrigel for 6 hour with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and DHA (30 µM) or BSA control. Scale bar, 500 µm. B, DHA did not reverse the inhibition of endothelial cell tubule formation by CYP2C inhibition. C, Montelukast had no significant effect on CYP2C8 mRNA levels in HRMECs. n = 6. * P < 0.05; ** P < 0.01; n. s., not significant.
**Supplemental Figure VII.** 19,20-EDP reversed the inhibition of HRMEC migration by CYP2C inhibition. A, Representative photos of HRMECs 24 hour after a scratch treated with the CYP2C inhibitor montelukast (20 µg/ml) or 0.2% DMSO control, and 19,20-EDP (1 µM) or ETOH vehicle control. Scale bar, 500 µm. B, 19,20-EDP rescued the inhibition of cell migration by CYP2C inhibition. n = 5. *** P < 0.001. C, Montelukast impaired VEGF-induced ERK phosphorylation in HRMECs.
Supplemental Figure VIII. CYP2C8, but not CysTLR1, deficiency impaired HRMEC tubule formation. A, Representative photos of HRMECs grown on Matrigel for treated for 6 hours with CYP2C or CysTLR1 siRNAs (10nM). Scale bar, 500 µm. B, CYP2C8, but not CysTLR1, siRNAs inhibited HRMEC tubule formation. C, CYP2C8, but not CysTLR1, siRNAs decreased CYP2C8 mRNA levels. CysTLR1 mRNA levels were undetectable in HRMECs. n = 6. *** P < 0.001; n. s., not significant.
Supplemental Figure IX. Montelukast lowered Vegfa but not Vegfc mRNA levels in mouse retina and choroid. Tie2-driven CYP2C8 transgenic (Tg) mice and wild-type (WT) littermate controls fed with a ω-3 LCPUFA enriched diet were intraperitoneally injected with the CYP2C inhibitor montelukast (1 mg/kg) or 10% DMSO control daily from P12 to P16 for OIR mice (A & B) or from day 0 to day 6 after laser photocoagulation for CNV mice (C & D). Montelukast reversed the induction of mRNA levels of Vegfa (A & C) but had no effect on Vegfc (B & D) expression in P17 OIR retina (A & B) or CNV choroid (C & D) 7 days after laser photocoagulation. n = 6 eyes/group. * P < 0.05; n. s., not significant.
Supplemental Table I. Plasma levels (pg/ml) of other LCPUFA metabolites in WT and CYP2C8 Tg mice fed with a ω-3 LCPUFA enriched diet and treated with the CYP2C8 inhibitor montelukast or DMSO as control.

<table>
<thead>
<tr>
<th>Metabolites measured</th>
<th>WT DMSO</th>
<th>CYP2C8 Tg DMSO</th>
<th>CYP2C8 Tg Montelukast</th>
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<tbody>
<tr>
<td>19,20-DiHDPA</td>
<td>51770.33</td>
<td>67596.17*</td>
<td>37378.02†</td>
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<td>17,18-DHEQ</td>
<td>108423.0</td>
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<td>14,15-DHET</td>
<td>3505.881</td>
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<td>17,18-EEQ</td>
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<td>304.7631*</td>
<td>177.0491†</td>
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<tr>
<td>14,15-EET</td>
<td>84.66607</td>
<td>176.3169*</td>
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<td>5-HETE</td>
<td>3297.219</td>
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<td>20-HETE</td>
<td>3580.750</td>
<td>5422.105*</td>
<td>4838.885†</td>
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<td>PGE2</td>
<td>460.6699</td>
<td>366.2372*</td>
<td>497.6818†</td>
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<tr>
<td>TXB2</td>
<td>2370.684</td>
<td>1819.801*</td>
<td>2549.474†</td>
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* significant difference vs. WT DMSO group
† significant difference vs. CYP2C8 Tg DMSO group

DHEQ, dihydroxy-eicosatetraenoic acid
EEQ, epoxyeicosatetraenoic acid
HETE, hydroxyeicosatetraenoic acid
PGE2, prostaglandin E2
TXB2, thromboxane B2
Supplemental Table II. Plasma levels (pg/ml) of other LCPUFA metabolites in WT and CYP2C8 Tg mice fed with a ω-6 LCPUFA enriched diet and treated with the CYP2C8 inhibitor montelukast or DMSO as control.

<table>
<thead>
<tr>
<th>Metabolites measured</th>
<th>WT DMSO</th>
<th>CYP2C8 Tg DMSO</th>
<th>CYP2C8 Tg Montelukast</th>
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<td>16803.31†</td>
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<td>TXB2</td>
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* significant difference vs. WT DMSO group
† significant difference vs. CYP2C8 Tg DMSO group
Supplemental Table III. Plasma levels of LCPUFA metabolites in WT C57BL/6 mice fed with normal feed and treated with the sEH inhibitor 1770 or DMSO as control.

<table>
<thead>
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<th>Metabolites measured</th>
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<th>sEH inhibitor 1770</th>
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<td>19,20-EDP</td>
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<td>17,18-EEQ</td>
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* significant difference vs. DMSO group