Beyond VEGF: targeting inflammation and other pathways for treatment of retinal disease

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Abbreviations:

AAV, adeno-associated virus (AAV); AMD, age-related macular degeneration; AP, apurinic/apyrimidinic; AP-1, activator protein 1; APE1, apurinic/apyrimidinic endonuclease 1; ARA, arachidonic acid; ARPE-19, adult retinal pigment epithelial cell line-19; ATP, adenosine triphosphate; BER, base excision repair; BID, twice daily; CB2, cannabinoid type 2; CBF, core-binding factor; CCL2, chemokine (C-C motif) ligand 2; CCR3, C-C motif chemokine receptor 3; CNV, choroidal neovascularization; CXCR4, chemokine CXC receptor-4; CYP, cytochrome P450; DHA, docosahexaenoic acid;
DHDPs, dihydroxydocosapentaenoic acids; DMI, diabetic macular ischemia; DME, diabetic macular edema; DR, diabetic retinopathy; DRSS, diabetic retinopathy severity scale; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; EDPs, epoxydocosapentaenoic acids; EETs, epoxyeicosatrienoic acids; EEQ, 17,18-epoxyeicosatetraenoic acid; EpFAs, epoxygenated fatty acids; ETC, electron transport chain; FDA, Food and Drug Administration; FA, fluorescein angiography; FECH, ferrochelatase; FVM, fibrovascular membrane; GCL, ganglion cell layer; HIF-1α, hypoxia inducible factor 1α; HRECs, human retinal microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; iCEC2, induced pluripotent stem cell-derived choroidal endothelial cell line; IGF, insulin-like growth factor; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-18; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-18, interleukin-18; INL, inner nuclear layer; IP, intraperitoneal injection; IVT, intravitreal injection; JNK, c-Jun N-terminal kinase; L-CNV, laser-induced choroidal neovascularization; ΔΨm, mitochondrial membrane potential; nAMD, neovascular age-related macular degeneration; NF-κB, nuclear factor κ light-chain-enhancer of activated B cells; NLRP3, inflammasome nucleotide-binding oligomerization domain like receptor containing domain 3; NMPP, N-methylprotoporphyrin; O-GlcNAc, O-linked N-acetylglucosamine; NPDR, non-proliferative diabetic retinopathy; OCT, optical coherence tomography; ONL, outer nuclear layer; PD-L1, programmed cell death ligand-1; PDR, proliferative diabetic retinopathy; PPIX, protoporphyrin IX; PRMT5, protein arginine methyltransferase-5; PUFA, polyunsaturated fatty acid; PVR, proliferative vitreoretinopathy; Ref-1, reduction-oxidation factor 1; Rf/6a, macaque choroidal endothelial cell-like cell line; RNV, retinal neovascularization; ROCK, rho-
associated protein kinase; ROP, retinopathy of prematurity; ROS, reactive oxygen species; RPE, retinal pigment epithelium; RUNX1, runt-related transcription factor 1; sEH, soluble epoxide hydrolase; SDF-1, stromal derived factor-1; shRNA, small hairpin RNA; STAT3, signal transducer and activator of transcription 3; t-AUCB, trans-4-(4-(3-adaman-1-yl-ureido)-cyclohexyloxy)-benzoic acid; TF, transcription factor; Tff1, trefoil factor family 1; TGF-β2, transforming growth factor-β2; TNF-α, tumor necrosis factor-alpha; TPPU, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; YAP, yes-associated protein.

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Abstract

Neovascular eye diseases include conditions such as retinopathy of prematurity, proliferative diabetic retinopathy, and neovascular age-related macular degeneration. Together, they are a major cause of vision loss and blindness worldwide. The current therapeutic mainstay for these diseases is intravitreal injections of biologics targeting vascular endothelial growth factor (VEGF) signaling. Lack of universal response to these anti-VEGF agents coupled with the challenging delivery method underscore a need for new therapeutic targets and agents. In particular, proteins that mediate both inflammatory and proangiogenic signaling are appealing targets for new therapeutic development. Here, we review agents currently in clinical trials and highlight some promising targets in preclinical and early clinical development, focusing on the redox-regulatory transcriptional activator APE1/Ref-1, the bioactive lipid modulator soluble epoxide hydrolase (sEH), the transcription factor RUNX1, and others. Small molecules targeting each of these proteins show promise for blocking neovascularization and inflammation. The affected signaling pathways illustrate the potential of new antiangiogenic strategies for posterior ocular disease.

Significance Statement

Discovery and therapeutic targeting of new angiogenesis mediators is necessary to improve treatment of blinding eye diseases like retinopathy of prematurity, diabetic retinopathy, and neovascular age-related macular degeneration. Novel targets undergoing evaluation and drug discovery work include proteins important for both angiogenesis and inflammation signaling, including APE1/Ref-1, soluble epoxide hydrolase, RUNX1, and others.
Introduction: The Challenge of Neovascular Eye Diseases

Neovascularization, or aberrant blood vessel growth, is a defining phenotype of an array of blinding eye diseases. Major neovascular posterior eye diseases include retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and the neovascular form of age-related macular degeneration (nAMD). Together, these are major contributors to blindness worldwide. ROP is among the most common causes of vision loss in children, with more than 30,000 estimated cases annually worldwide (Hong et al., 2022). Diabetic retinopathy (DR) affects 35% (Yau et al., 2012) of the growing diabetic population, which is now estimated at more than half a billion people globally (International Diabetes Federation, 2021). PDR affects about 7%, and diabetic macular edema affects 7% (Yau et al., 2012). nAMD, while only accounting for about 10% of the more than 200 million total AMD patients worldwide (Wong et al., 2014), is responsible for 90% of the AMD-related blindness (Ferris et al., 1984).

ROP develops in premature infants exposed to postnatal hyperoxia, which pauses retinal vasculature development (Phase 1). On return to normoxia, aberrant, leaky preretinal neovascularization develops (Phase 2) (Fevereiro-Martins et al., 2022) (Figure 1). This disease is modeled quite faithfully in the oxygen-induced retinopathy (OIR) rodent model involving postnatal hyperoxia exposure (Smith et al., 1994). In DR, hyperglycemia initially leads to vascular dysfunction; inflammation characterized by gliosis, microglial activation, and edema (Rübsam et al., 2018); and ischemic areas. In some cases, this is followed by aberrant compensation in the form of retinal neovascularization, a hallmark of PDR (Antonetti et al., 2021) (Figure 1). The OIR model, as an ischemic retinopathy model, is also often used as a surrogate in PDR...
research, given that hyperglycemia-driven PDR animal models are not widespread (Antonetti et al., 2021). Both non-proliferative DR and PDR in humans can be associated with DME, fluid leakage leading to retinal thickening (Antonetti et al., 2021) (Figure 1). Finally, nAMD develops from the “dry” form of AMD, a chronic disease of aging characterized by lipid-rich deposits (drusen), and photoreceptor and retinal pigment epithelium (RPE) degeneration (Fleckenstein et al., 2021). In some cases, dry AMD progresses to nAMD, when macular neovascularization, usually arising from the choroid (choroidal neovascularization, CNV) invades the outer retina (Fernandes et al., 2022) (Figure 1). A widely used model with features of nAMD is laser-induced CNV (L-CNV) in rodents or primates, in which a laser burn disrupts Bruch’s membrane that separates the choroid from the RPE, promoting an acute neovascular response (Grossniklaus et al., 2010; Malek et al., 2018).

The insults that drive neovascularization in these diseases are varied: largely ischemia/hypoxia (in ROP and PDR) or largely oxidative stress/inflammation (in nAMD), but in most cases, the eventual proangiogenic stimulus is provided by the VEGF signaling cascade (Ramakrishnan et al., 2014; Apte et al., 2019). Since the approval of the first anti-VEGF ocular agent, pegaptanib (an aptamer), almost two decades ago (Gragoudas et al., 2004), therapy of these neovascular eye diseases has been revolutionized (Furino et al., 2021). The current anti-VEGF agents approved by the FDA for various neovascular eye disease indications include ranibizumab (antibody) (Folk and Stone, 2010), aflibercept (fusion protein) (Do et al., 2011), brolucizumab (antibody) (Dugel et al., 2020), and most recently faricimab (dual-targeting antibody that also targets angiopoietin signaling) (Heier et al., 2022; Wykoff et al., 2022). Bevacizumab,
the prototypical anti-VEGF antibody, is also widely used off-label (Tufail et al., 2010). In addition, there are multiple new candidate drugs currently in different phases of clinical trials, primarily targeting VEGF (Table 1). These include KSI-301, an antibody-polymer conjugate from Kodiak; RGX-314, an anti-VEGF gene therapy from REGENXBIO; and two tyrosine kinase inhibitor formulations blocking VEGFR2 (and other receptor) activities (EYP-1901 from EyePoint and OTX-TKI from Ocular Therapeutix). BI 764524 from Boehringer Ingelheim is an exception to this anti-VEGF trend: it is an antibody that targets semaphorin 3A, a vasorepulsive axon guidance cue; semaphorin 3A inhibition may ameliorate diabetic macular ischemia (Zippel et al., 2022).

Although the existing approved agents have greatly improved outcomes for patients with ROP, PDR, nAMD, and other neovascular eye diseases, they are not without their shortcomings. Anti-VEGF biologics can lead to tachyphylaxis, resistance due to compensatory mechanisms via usage of alternative angiogenic pathways, tolerance, and systemic side effects (Stewart, 2012; Yang et al., 2016; Ricci et al., 2020). Real-world responses have not lived up to the promise of the registration trials, largely due to less frequent dosing outside the highly regimented context of clinical trials (Hsu and Regillo, 2020; Mehta et al., 2022). And delivery is a significant challenge: all the currently approved agents must be intravitreally (IVT) injected, with the exception of a recently approved refillable port delivery system for ranibizumab (Susvimo) that is surgically implanted (Holekamp et al., 2021; Adamis and de Juan, 2022). IVT injections must be done by an ophthalmologist and have a small but significant risk of serious complications such as endophthalmitis (Day et al., 2011; Patel et al., 2022). Moreover, understandably, these injections are unpopular with patients; 80% of patients in one
study stated a preference for oral or topical therapies, if such were available (Jacobs et al., 2021). And this is not only a question of preference: the time, travel, and cost required for appointments for IVT injections can lead to non-compliance or outright lack of access to therapy for some individuals and in low-resource settings (Weiss et al., 2018; Bascaran et al., 2021; Okada et al., 2021).

Given these shortcomings, there continues to be substantial interest in developing new approaches for treating ocular neovascularization. Especially intriguing are efforts to move beyond directly regulating VEGF signaling and/or to impinge on the multiplex signaling pathways that drive angiogenesis, including inflammation, hypoxia, oxidative stress, and other pathways. In this minireview, we highlight some of the approaches currently in clinical trials, then focus on novel targets under investigation in our laboratories and others to illustrate the diversity of targets that may hold promise for future neovascular eye disease therapies (Figure 1).

New Targets (non-VEGF) in Clinical Trials: Links to Inflammation

Inflammatory signaling pathways are highly active in ocular settings, which results in a wide range of ocular inflammatory diseases, including DR and nAMD (Kim et al., 2021). Prolonged and exacerbated local inflammation in the eyes is directly or indirectly a major cause of vision impairment or blindness. For instance, inflamed eyes in some cases can develop CNV or retinal neovascularization (RNV), which may be successfully treated with anti-inflammatory agents alone. Inflammation responses such as oxidative stress, leukocyte migration and lipid deposition lead to RPE and photoreceptor degeneration in nAMD by increasing the cell permeability and the production of angiogenic factors (Carmi et al., 2009; Xu et al., 2009; Toomey et al., 2018). Advances
in the understanding of inflammatory processes have revealed new key pathways, such as VEGF and molecular factors involved in the mechanisms of inflammation.

Anti-VEGF injections do not completely meet patients’ needs due to the multifactorial nature of retinal diseases, which may include an inflammatory component. Furthermore, long-term administration of IVT anti-VEGF injections could increase the risk of developing retinal scarring and could result in other complications such as vitreous or subconjunctival hemorrhage and inflammation in the eyes (Daniel et al., 2014; Yerramothu, 2018).

Multiple signaling pathways such as the complement pathway, stromal derived factor-1 (SDF-1)/chemokine CXC receptor-4 (CXCR4), inflammasome nucleotide-binding oligomerization domain-like receptor containing domain 3 (NLRP3), interleukin 18 (IL-18), programed cell death ligand-1 (PD-L1), insulin-like growth factor (IGF) and Yes-associated protein (YAP) signaling pathways were recently implicated in the pathogenesis of DR, nAMD and ocular inflammation (Wu et al., 2017; Yan et al., 2018; Yerramothu, 2018; Kim et al., 2021). However, some of these signaling pathways work together with upstream or downstream effectors of VEGF signaling to some extent, such as the complement pathway, transforming growth factor-β (TGF-β) signaling, as well as activation of crucial oncogenic transcriptional factors (TFs) such as NF-κB and STAT3 (Wang et al., 2017; Sardar Pasha et al., 2018; Dong et al., 2020). Complement signaling has a potential role in the resolution phase of inflammation. For example, both complement components C5a and C3a stimulate VEGF expression in post-injury angiogenesis and could also be involved in CNV (Nozaki et al., 2006; Grambergs et al., 2019).
As mentioned above, several targets are being pursued for non-VEGF ocular inflammation treatments and are at the clinical trial stage (Table 2), notably for DR, DME and nAMD. Runcaciguat (BAY1101042) is a soluble guanylate cyclase (sGC; aids in maintaining the retinal vasculature homeostasis) activator developed by Bayer, as an oral clinical candidate in a Phase 2 clinical trial for NPDR (NCT04722991) (Hahn et al., 2021).

AKST4290 is an oral drug developed by Alkahest, designed to block the chemokine eotaxin from binding to its receptor, C-C motif chemokine receptor 3 (CCR3) as a novel oral therapy for inflammation-mediated nAMD (NCT04331730) (Sharma et al., 2012; Hirahara et al. 2017; Stewart et al., 2022). Vicasinabin (RG7774) is a cannabinoid 2 receptor (CB2R) agonist that could modulate pro- and anti-inflammatory signaling in cells, developed by Roche for the treatment of DR (NCT04265261).

Xiflam/tonabersat (HCB1019) is a blocker of connexin hemichannels, such as connexin 43 (a gap junction protein that facilitates secretion of inflammatory cytokines) and the NLRP3 inflammasome. It is currently in Phase 2 clinical trials for treating DME (Danesh-Meyer et al. 2016; Mat Nor et al., 2020; Lyon et al., 2021) (NCT05727891). OPL-0401 is a small molecule Rho kinase 1/2 (ROCK; plays a key role in endothelial cell migration induced by VEGF; Arita et al. 2010) inhibitor from Valo that is a potential first-in-class oral drug for NPDR (NCT05393284). RZ402 is a selective and potent plasma kallikrein inhibitor (PKI) developed by Rezolute for the treatment of DME (NCT05712720).
OCS-01 from Oculis is a dexamethasone eyedrop being tested in DME (NCT05066997), while OTT166/SF0166 is a novel small molecule selective integrin (αvβ3) inhibitor designed to reach the retina via topical administration for the treatment of DME (Askew et al. 2018; Boyer et al., 2022) (NCT02914613).

Finally, APX3330 is a novel oral agent that targets APE1/Ref-1, an attractive molecular target alleviating inflammatory burden in ocular settings. This target is discussed more in the following section (Hartman et al., 2021; Heisel et al., 2021). Most of the above agents have completed Phase 1 trials and are in Phase 2 (Table 2). All the candidates discussed above are given orally except for OCS-01 and OTT166 which are eyedrops. Anti-VEGF combination therapies could provide significant improvement in overall treatment outcomes. This would include compounds targeting inflammation coupled with anti-VEGF therapy in the appropriate disease, to afford an increased efficacy and potentially increased time between treatments. Additionally, use of agents that have anti-VEGF effectiveness but delivered in a route independent of IVT injection, by eyedrops or systemically, could significantly improve treatment outcomes. This approach could have many benefits: increased efficacy, reduced trips for injections, and potential cost savings over a longer period of time, resulting in multiple benefits from the advancement of new targets involved in ocular diseases.

New Targets in Development

Apurinic/apyrimidinic endonuclease 1/reduction-oxidation factor 1 (APE1/Ref-1)

APE1/Ref-1 (gene name: APEX1) is one of several novel targets in development for treating retinal diseases. This multifunctional protein possesses both DNA repair activity
(APE1) and a redox-transcription regulation role (Ref-1) and has been linked to pathways involved in several retinal disease states, including PDR, ROP, and nAMD (Heisel et al., 2021; Mijit et al., 2021). The DNA repair activity of APE1 regulates both short and long patch base excision repair (BER) by hydrolyzing the phosphodiester backbone of an abasic site, enabling DNA polymerase to integrate new nucleotides (Caston et al., 2021). The repair of oxidative DNA damage is a major focus of APE1/Ref-1 and the BER pathway. The redox activity of Ref-1 regulates TFs that play a role in driving angiogenesis and inflammation, including HIF-1α, NF-κB, and STAT3 (Heisel et al., 2021; Mijit et al., 2021). By chemically reducing critical cysteine residues, Ref-1 allows the TFs to bind to DNA and influence gene expression and protein synthesis, thereby allowing for the activation of key pathways involved in neovascular eye diseases such as inflammation, angiogenesis, oxidative stress response, and cell survival pathways (Kelley et al., 2012; Shah et al., 2017) (Figure 2A). Additionally, in a recently completed study using RNA-seq analysis following knockdown of APEX1 in human retinal endothelial cells (HRECs), we identified multiple downregulated genes. These were involved in DNA base excision repair, other DNA repair pathways, purine or pyrimidine metabolism signaling, and histidine/one carbon metabolism pathways. This contrasts with APEX1 knockdown data in multiple human cancer cell lines and highlights the distinctive role of Ref-1 in the eye and possible ocular therapeutic opportunities (Mijit et al., 2023).

Ref-1 redox activity is essential for angiogenesis, and there is strong in vitro evidence that Ref-1 redox inhibitors suppress angiogenesis in several cell types. APX3330, a dimethoxy benzoquinone, is a small molecule redox inhibitor of Ref-1 that
increases oxidation of the active disulfide residues in Ref-1 to block activation of key TFs without inhibiting the DNA repair activity (Luo et al., 2008; Fishel et al., 2010; Fishel et al., 2011; Jedinak et al., 2011; Su et al., 2011; Cardoso et al., 2012; Zhang et al., 2013b; Fishel et al., 2015; Choi et al., 2016; Shah et al., 2017). APX3330 dose-dependently decreases cell proliferation, migration, and tube formation in HRECs and a macaque choroidal endothelial cell-like cell line (Rf/6a), demonstrating the potent anti-angiogenic activity of the small molecule inhibitor (Jiang et al., 2011; Li et al., 2014b; Sardar Pasha et al., 2018). NF-κB, a TF known to activate genes involved in inflammation, proliferation, migration, and invasion, exhibits decreased activity in response to APX3330 (Hiramoto et al., 1998; Li et al., 2014a). APX3330 also reduces STAT3 DNA binding, reduces intracellular ROS levels, and protects cells from senescence (Luo et al., 2008; Li and Wilson, 2014; Li et al., 2014a; Sardar Pasha et al., 2018). Furthermore, inhibition of Ref-1 with APX3330 produces an anti-inflammatory response and alleviates oxidative stress in a mouse model of inflammatory bowel disease (IBD), suggesting that Ref-1 mediates inflammation signaling (Sahakian et al., 2021). APX3330 does not contribute to apoptosis and even provides neuronal protection, primarily from oxidative DNA damage, by enhancing the AP endonuclease repair activity of APE1/Ref-1 (Fehrenbacher et al., 2017; Sahakian et al., 2021).

In vivo, APX3330 shows promising therapeutic effects in several mouse models, suggesting Ref-1 to be a suitable target for treatment of neovascular eye diseases. One IVT injection of APX3330 decreased neovascularization in the Vldlr−/− mouse model, a model of subretinal neovascularization (Jiang et al., 2011). IP injection of APX3330 twice daily for two weeks decreased L-CNV lesion volume by 25% (Sardar Pasha et al.,
2018) and reduced lesion size by 50% when delivered via gavage in the L-CNV model (Hartman et al., 2021; Lachi Silva et al., 2021). This is comparable to IVT injections of anti-VEGF antibody, which decrease lesion size by 30-50% in the L-CNV model (Sulaiman et al., 2016). These considerable findings and the oral bioavailability of APX3330 illustrate that Ref-1 redox inhibitors may be a more effective treatment option for retinal diseases than current regimens by targeting multiple pathways involved in retinal diseases.

Like some current approved anti-VEGF therapeutics, APX3330 has an oncology origin and was originally developed by Eisai for multiple hepatic inflammatory indications. To date, APX3330 has completed 12 phase 1 and phase 2 clinical trials and has been extensively investigated in vitro and in vivo, yielding positive safety and efficacy findings. Efforts to develop second-generation Ref-1 redox inhibitors are already underway, including compounds APX2009 and APX2014. These second-generation Ref-1 redox inhibitors have reduced lipophilicity than APX3330, thus increasing efficacy and possibly bioavailability (Kelley et al., 2011; Sardar Pasha et al., 2018). These small molecules effectively reduce endothelial cell proliferation, migration, and tube formation, and IP APX2009 effectively reduces lesion size in the L-CNV mouse model (Sardar Pasha et al., 2018).

APX3330’s ability to regulate multiple TFs regulating pro-angiogenic and pro-inflammatory pathways makes it an attractive therapeutic for eye diseases. In addition, its ability to promote repair of oxidative damaged DNA by APE1/Ref-1 adds a unique component. Thus, it has undergone clinical evaluation. The ZETA-1 trial was a 24-week, randomized, placebo-controlled, double-masked study in which patients received twice
daily oral tablets 300 mg (600 mg/day) APX3330 or placebo to evaluate the safety and efficacy of APX3330 for patients with DR and DME (NCT4692688). APX3330 achieved statistical significance on a secondary endpoint of preventing clinically meaningful progression of DR, as defined by binocular 3 or more steps worsening on the DRSS. Prevention of 3-step worsening (binocular) is a suitable endpoint for an oral, systemic drug. Following confirmation of this as a potential registration endpoint with the FDA for a Phase 3 trial for DR, a Phase 3 meeting is planned. The safety results from the ZETA-1 trial also demonstrate that APX3330 as an oral drug remains excellent and consistent what was previously reported (Boyer et al., 2022).

Because Ref-1 redox activity regulates multiple TFs that are linked to neovascular eye diseases, it may be a more promising therapeutic target than current treatments that only target the VEGF signaling pathway. The targets of APX3330 are validated retinal disease pathways, and extensive in vitro and in vivo validation demonstrate the specificity and effectiveness of APX3330 with favorable human safety data (Boyer et al., 2022). With current treatments consisting of invasive IVT injections, an orally bioavailable drug such as APX3330 or other Ref-1 redox inhibitors offer a non-invasive route compared to standard of care IVT injections. Together, this evidence suggests that targeting Ref-1 for neovascular eye diseases may address several clinical problems with current approved therapeutics.

**Soluble epoxide hydrolase (sEH)**

Polyunsaturated fatty acid (PUFA) metabolism, specifically targeting the function of sEH (gene name: Ephx2) is another promising area for combined antiangiogenic/anti-
inflammatory therapy in neovascularization. Docosahexaenoic acid (DHA) is a PUFA and one of the major components of the retina (makes up ~60% of the fatty acids in the photoreceptors) while the abundance of DHA in other tissues is much lower (~5% of total fatty acids) (Stinson et al., 1991; Bush et al., 1994; Stillwell and Wassall, 2003; Querques et al., 2011). DHA can be epoxidated to epoxydocosapentaenoic acids (EDPs) by cytochrome P450 (CYP). Hydrolysis via sEH generates a group of metabolites called dihydroxydocosapentaenoic acids (DHDPs) which lead to proangiogenic phenotypes (Figure 2B) (Arnold et al., 2010a; Harris and Hammock, 2013).

CYP is responsible for converting PUFAs to bioactive epoxygenated fatty acids (EpFAs). Specifically, CYP targets the ω-6 double bond of arachidonic acid (ARA) to produce epoxyeicosatrienoic acids (EETs). CYP also targets the ω-3 double bond of DHA, with a higher catalytic efficiency than ARA (Arnold et al., 2010a), and produces EDPs (Fer et al., 2008; Arnold et al., 2010b). Both metabolized EpFAs have been studied in retinopathy due to their functions of vasodilation and anti-inflammation (Ye et al., 2002; Zhang et al., 2014; Capozzi et al., 2016).

EpFAs, such as EETs and EDPs, are unstable and can be rapidly metabolized by enzymes, mainly sEH (Chacos et al., 1983). 19,20-EDP is the most abundant ω-3 EpFA isomer since it is the least efficient substrate for sEH (Zhang et al., 2014). Inhibition of sEH stabilizes and enhances bioactivities of EpFAs, and while EETs are thought to promote angiogenesis (Oltman et al., 1998; Zhang et al., 2001; Ye et al., 2002), EDPs are antiangiogenic in some studies (Zhang et al., 2013a; Capozzi et al., 2014; Hasegawa et al., 2017; Hu et al., 2017). Thus, sEH inhibitors are thought to be
antiangiogenic in the eye as the result of accumulation of EDPs after sEH inhibition (Sulaiman et al., 2016; Sulaiman et al., 2018). We recently showed that IVT delivery of 19,20-EDP reduces L-CNV, while 19,20-DHDP has no effect (Park et al., 2022). However, conflicting results have been observed in some other studies: Tie2-driven sEH-overexpressing mice experience reduced CNV (Gong et al., 2017). And a proangiogenic role of 19,20-EDP was seen in OIR (Shao et al., 2014). As reviewed previously (Park and Corson, 2019), different experimental setups possibly contribute to these contradictory findings. Systemic vs. tissue specific inhibition of sEH (oral administration/IP injection vs. tissue specific inhibition) might have different effects considering the unique retinal lipid composition. In addition, administering 19,20-EDP without sEH inhibitors may not only increase EDP but also increase 19,20-DHDP by sEH. Thus, the observed angiogenesis with 19,20-EDP treatment could be potentially due to the increased 19,20-DHDP.

Dietary PUFAs have been extensively studied as antiangiogenic therapies in animal models such as L-CNV. Anti-inflammatory and anti-angiogenic effects were observed by providing ω-3 PUFAs but not ω-6 PUFAs in the diet of this model (Yanai et al., 2014). Providing 17,18-epoxyeicosatetraenoic acid (EEQ) and 19,20-EDP instead of ω-3 PUFAs in the diet of L-CNV mice also decreased CNV, suggesting that this protective effect was regulated by PUFAs' downstream metabolites catalyzed by CYP (Yanai et al., 2014). Exogeneous ω-3 PUFAs in the diet also reduced lesion size in L-CNV mice with Ephx2 knockout, which further confirmed the sEH dependent metabolism of 17,18-EEQ and 19,20-EDP (Hasegawa et al., 2017). However, due to varying compositions of ω-3 PUFAs in different tissues, systemic administration of
dietary PUFAs might have unexpected effects as noted above. Thus, treatment localized to the eye for neovascular eye diseases may be a promising therapeutic strategy.

In the retinas of L-CNV mice, sEH was elevated in the photoreceptors and enzyme activity increased in adult mice after laser induction (Sulaiman et al., 2018). Significantly decreased 19,20-EDP:19,20-DHDP ratio in retinas of L-CNV mice implicated increased sEH activity (Sulaiman et al., 2018). Recently, both immunohistochemistry and RNAscope in situ hybridization data indicated that sEH was overexpressed in photoreceptors and RPE in nAMD human retinas and L-CNV mouse retinas (Park et al., 2022).

Small molecule sEH inhibitors are effective in reducing ocular neovascularization in mouse models. We developed a synthetic homoisoflavonoid sEH inhibitor (Sulaiman et al., 2018), SH-11037, that inhibits the growth and migration of HRECs, without cytotoxicity (Basavarajappa et al., 2015), and is antiangiogenic in both the choroidal sprouting assay ex vivo and in zebrafish larvae (Sulaiman et al., 2016). In addition, IVT injection of SH-11037 reduced CNV lesions in the L-CNV model in vivo (Sulaiman et al., 2016) and in OIR (Basavarajappa et al., 2015). Similar results were seen in L-CNV with other sEH inhibitors t-AUCB and “compound 7” (Sulaiman et al., 2018). The lipid composition of the mouse eye could be altered effectively by IVT injection of 10 µM SH-11037 or t-AUCB – the 19,20-EDP:19,20-DHDP ratio increased (Sulaiman et al., 2018). sEH inhibitor treatment by other administration routes has also been reported. t-AUCB in drinking water (2 mg/L) significantly reduced 19,20-DHDP in diabetic mouse retina and rescued vascular defects (reduced pericyte number, increased vascular
permeability), which further showed a protective effect of sEH inhibitors (Hu et al., 2017). These results indicated that sEH could also be relevant to non-proliferative DR. Orally dosed 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), an sEH inhibitor that can pass through the blood-brain barrier co-administrated with 17,18-EEQ or 19,20-EDP (IP) suppressed CNV (Hasegawa et al., 2017), while t-AUCB given to normal neonatal mice (IP injection, 2 mg/kg, BID) reduced retinal vascularization significantly (Hu et al., 2014). Our recent work showed that adeno-associated virus (AAV) serotype 8 vector expressing shRNA against Ephx2 decreased L-CNV lesion volume when delivered IVT (Park et al., 2022). Ephx2 shRNA also decreased expression of inflammatory cytokines (IL-1β, IL-6, TNF-α etc.) (Park et al., 2022). Taken together, these results confirm that acute inflammation is tightly related with CNV progression, especially in the early stage (Choudhary and Malek, 2019), and that sEH inhibition can ameliorate this. Based on these fundamental research results about the metabolism of the CYP-sEH pathway and the effects of sEH inhibition on inflammation and neovascularization, sEH could be a potential target against ocular neovascularization and DR.

**Runt-related transcription factor 1 (RUNX1)**

A protein that controls the development of blood vessels, RUNX1, is another novel target under exploration for varied ocular neovascular pathologies. RUNX1 (also known as AML1) is a transcription factor that was initially identified to regulate hematopoiesis in acute myeloid leukemia (AML) patients (Miyoshi et al., 1991). Core-binding factor (CBF) protein is a heterodimeric protein complex wherein RUNX1 forms the α-subunit responsible for DNA binding and activation of transcriptional targets while
the CBFβ subunit stabilizes this complex in addition to increasing binding affinity of DNA (Lam et al., 2017) (Figure 2C). RUNX1 is upregulated in numerous cancers including glioblastoma (Zhao et al., 2019), and this protein regulates angiogenic cell migration, proliferation, cell differentiation, invasion, tubule formation, and other phenotypes (Lam et al., 2017). As such, RUNX1 is implicated in various ocular pathologies including PDR (Lam et al., 2017), proliferative vitreoretinopathy (PVR) (Delgado-Tirado et al., 2020), and CNV (Gonzalez-Buendia et al., 2021). In PVR, which occurs after rhegmatogenous retinal detachment, RUNX1 promotes epithelial to mesenchymal transition of RPE cells facilitated through TGF-β2 signaling. Plus, PVR tissue specimens have high expression of RUNX1 (Delgado-Tirado et al., 2020). In PDR, fibrovascular membrane (FVM)-derived CD31+ vascular endothelial cells show aberrant upregulation of RUNX1. In L-CNV, RUNX1 is highly expressed in the lesion area and in cell types contributing to neovascularization, including endothelial cells, macrophages, microglia, RPE cells, Müller cells and vascular smooth muscle cells (Gonzalez-Buendia et al., 2021).

Further, in HRECs and human umbilical vein endothelial cells (HUVECs), RUNX1 protein increases in response to high glucose conditions and regulates HREC migration, proliferation, and tubule formation (Lam et al., 2017). Since high glucose can modulate RUNX1, the involvement of RUNX1 in high glucose-induced post-translational modification of O-linked N-acetylglucosamine (O-GlcNAc) also promotes HREC proliferation and migration (Xing et al., 2021) in DR. Activation of p38 MAPK causes RUNX1 upregulation and RUNX1-dependent abnormal angiogenic properties in HRECs and in a mouse model of streptozotocin-induced DR (Zou et al., 2020). As added evidence, RUNX1 regulates the transcriptional activation of Trefoil factor family 1 (Tff1)
peptide to repress this molecule in DR via dysregulating NF-κB signaling (Zhang et al., 2022). These studies show that RUNX1 plays a key role in ocular angiogenesis through multiple signaling pathways, which opens an avenue for the development of novel therapeutics. These links have generated increased attention in recent years to identify small molecule inhibitors to modulate RUNX1-mediated angiogenesis in the eye.

To inhibit RUNX1’s function, a specific lipophilic inhibitor of its transcriptional activation, Ro5-3335, has been widely used in vitro and in vivo. Both siRNA knockdown of RUNX1 and Ro5-3335 treatment in HRECs reduces angiogenic properties (Lam et al., 2017). Interestingly, Ro5-3335 dose-dependently reduced the proliferation of a primary culture established from PVR membranes. Upon inflammation, TNF-α activates c-Jun N-terminal kinase (JNK) signaling, which in turn accelerates RUNX1 transcription in HRECs. Ro5-3335 blocks RUNX1-mediated transcription (since this gene self-regulates), and so downregulates the mRNA expression of TNF-α induced RUNX1 and JNK in HRECs (Whitmore et al., 2021), suggesting the potential of Ro5-3335 for blocking TNF-α mediated inflammatory mechanisms in ocular angiogenesis. Beyond Ro5-3335, another small molecule inhibitor of RUNX1, Ro24-7429 as a nano-emulsion obstructs migration and proliferation in HRECs (Arevalo-Alquichire et al., 2022).

TNF-α and high glucose stimulate RUNX1 transcription through JNK and AP-1, which eventually builds a feedback loop of JNK-AP-1-RUNX1 transduction accountable for increased RUNX1 expression and angiogenesis progression. Intriguingly, VEGF modulates this feedback mechanism by inhibiting phosphorylation of JNK, and thereby repressing the expression of RUNX1 (Whitmore et al., 2021). However, the interaction between RUNX1 and VEGF in promoting angiogenesis needs to be validated further.
In vivo, inhibition of RUNX1 by topical application of Ro5-3335 nano-emulsion formulation impedes the progression of PVR in a rabbit model (Delgado-Tirado et al., 2020). RUNX1 inhibition through IVT injection of Ro5-3335 also ameliorates L-CNV, alone and in combination with aflibercept (Gonzalez-Buendia et al., 2021). Moreover, RUNX1 inhibition using IVT Ro5-3335 decreases neovascularization in murine OIR (Lam et al., 2017). No small molecule RUNX1 inhibitors have been in clinical use yet, but these findings indicate that targeting RUNX1 using Ro5-3335 holds promise for further validation in treating ocular neovascular diseases.

Other developing targets

In addition to the promising targets reviewed above, there are numerous other potential targets for ocular angiogenesis under exploration that are (at least partially) independent of the VEGF pathway, functioning through specific signaling pathways and targeted in preclinical work by small molecule inhibitors.

Protein arginine methyltransferase-5 (PRMT5). PRMT5, a type II arginine methyltransferase, and novel activator of NF-κB (Wei et al., 2014; Prabhu et al., 2017), has been identified as a novel target in ocular neovascularization (Muniyandi et al., 2023). PRMT5 methylates both histone and non-histone proteins thereby regulating their activities. In HRECs and cancer cells, PRMT5 can dimethylate the p65 subunit of NF-κB, promoting NF-κB activation and inflammatory cell signaling (Wei et al., 2013). A variety of cancers show overexpression of PRMT5 (Yan et al.; Han et al., 2014; Jiang et al., 2018; Zhang et al., 2018; Li et al., 2019; Qin et al., 2019), and we showed that PRMT5 is overexpressed in human nAMD and murine L-CNV and that it modulates proangiogenic and proinflammatory NF-κB signaling in HRECs. A novel, specific small
molecule inhibitor of PRMT5, PR5-LL-CM01, or shRNA knockdown dampened this signaling and angiogenesis properties in both HRECs and in an induced pluripotent stem cell-derived choroidal endothelial cell line (iCEC2) (Muniyandi et al., 2023).

**Ferrochelatase (FECH).** FECH controls the terminal step of heme biosynthesis, inserting ferrous iron (Fe$^{2+}$) into protoporphyrin IX (PPIX) to form heme (Hamza and Dailey, 2012) (Figure 2D). This heme acts as a co-factor for the hemoproteins in the cell (Smith et al., 2010). FECH is a target of the antiangiogenic natural product, cremastranone (Shim et al., 2004; Kim et al., 2007; Kim et al., 2008; Lee et al., 2014; Basavarajappa et al., 2017). FECH is overexpressed in human nAMD and mice with L-CNVD (Basavarajappa et al., 2017) and OIR (Pran Babu et al., 2020). Increased synthesis of heme in microvascular endothelial cells drives aberrant angiogenesis (Petrillo et al., 2018).

*In vitro*, FECH blockade in HRECs via siRNA knockdown and treatment with N-methylprotoporphyrin (NMPP) hampers proliferation, migration, and tube formation. In addition, genetic or chemical inhibition of FECH in ocular ECs downregulates the protein levels of known activators of angiogenesis including eNOS, HIF-1α, and VEGFR2 (Basavarajappa et al., 2017), plus mitochondrial complex IV, leading to disruption of mitochondrial morphogenesis, membrane potential loss, diminished glycolysis and oxidative phosphorylation (Shetty et al., 2020). Therefore, we hypothesized that targeting heme synthesis via FECH would offer novel therapeutic options (Shetty and Corson, 2020). We developed a first-in-class, drug-like small molecule FECH inhibitor, SH-17023 (4e). Strikingly, SH-17023 obstructs proliferation, migration, tube formation, and dose-dependently decreases the expression of COX-IV.
(subunit I) protein in HRECs. SH-17023 also inhibits proliferation in iCEC2 choroidal endothelial cells (Sishtla et al., 2022).

In vivo, mice with a partial loss of function point mutation in FECH (Fech<sup>m1Pas</sup>, functionally heme and iron deficient) have reduced L-CNV (Basavarajappa et al., 2017) and OIR (Pran Babu et al., 2020). Further, the antifungal drug griseofulvin (metabolized into a FECH inhibitor in vivo) attenuates L-CNV in mice (Basavarajappa et al., 2017). Intravitreal griseofulvin or NMPP reduced neovascular tuft formation and vasoobliteration without toxicity in OIR (Pran Babu et al., 2020). Likewise, novel FECH inhibitor SH-17023 reduced lesion volume in L-CNV (Sishtla et al., 2022). Thus, targeting FECH has significant therapeutic potential and might provide promising therapeutic options for ocular neovascular diseases.
Conclusions and Future Prospects

The outlook for new therapeutic approaches for posterior ocular neovascularization continues to be promising. We illustrate here just some of the many new approaches undergoing exploration (ElSheikh et al., 2022). Beyond the agents already in advanced clinical development, APE1/Ref-1 holds potential for targeting both angiogenesis and inflammation, taking advantage of APX3330, previously advanced as an anti-cancer drug with a good safety profile as an oral agent. Likewise, targeting sEH can suppress multiple proangiogenic/proinflammatory pathways and extensive efforts for eye disease and other indications have presented an array of potential small molecule inhibitors. RUNX1, as a transcription factor, can impinge on multiple pathways and is also a target of multiple small molecules. In addition, other candidates such as PRMT5 and FECH expand the arsenal of possible targets.

One key question for all these targets is the optimal delivery route for therapy. The safety and ocular uptake of APX3330 when delivered orally provides a strong rationale for pursuing this route for APE1/Ref-1 inhibition. The variability of sEH function in different tissues argues for local inhibition of this target, perhaps by eyedrops or sustained release IVT formulations. Sustained-release small-molecule inhibition of FECH has shown proof-of-concept in our hands (Chobisa et al., 2021). Avoiding systemic toxicity for targets like RUNX1 and PRMT5 may also necessitate local delivery, taking advantage of novel delivery systems like nano-emulsions (Arevalo-Alquichire et al., 2022), microparticles (Kim et al., 2019), polymeric implants (Wang et al., 2013), or suprachoroidal injections (Wan et al., 2021).
Much remains to be done in dissecting the molecular mechanisms of these targets as well, including determining whether they work with or through VEGF, and therefore their potential in combination therapy with anti-VEGF agents and/or their efficacy in poor- or non-responders to anti-VEGF drugs. The dearth of animal models for VEGF non-response hampers these efforts; synergy experiments may be helpful.

Finally, defining the diseases and disease subtypes most likely to benefit from inhibiting these targets also requires both pre-clinical and clinical work. If successful for (some) posterior neovascular eye diseases, there is appealing potential to explore these targets in anterior ocular neovascularization such as corneal neovascularization induced by injury or infection, which lacks widely effective therapies (Barry et al., 2020). Given the anti-inflammatory potential of targeting APE1/Ref-1 and sEH, assessing these in inflammatory diseases like uveitis or dry AMD is also appealing. With further preclinical and clinical testing, formulation, and mechanistic studies, one or more of these disease targets may move beyond VEGF to offer new therapeutic options for neovascularization in the eye.

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Figures were generated using biorender.com.

**Authorship Contributions**

*Performed data analysis:* Muniyandi, Hartman, Song, Mijit, Kelley, Corson

*Wrote or contributed to the writing of the manuscript:* Muniyandi, Hartman, Song, Mijit, Kelley, Corson
Disclosure of Potential Conflict of Interest

M.R.K. and T.W.C. are named inventors on patents related to this work, licensed to Apexian Pharmaceuticals and Ocuphire Pharma, or optioned to Evergreen Therapeutics. M.R.K. is a member of the Ocuphire medical advisory board and CSO and co-founder of Apexian Pharmaceuticals which developed APX3330 for oncology, as well as the other APX compounds listed in this manuscript. T.W.C. has received research funding and is a consultant for Evergreen Therapeutics. The other authors declare no conflicts of interest. None of Apexian Pharmaceuticals, Ocuphire Pharma, or Evergreen Therapeutics had any input or control over the contents of this manuscript.
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Footnotes

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Figure Legends

Figure 1. Pathologies of selected posterior ocular diseases (DR and DME, PDR, ROP, and nAMD) and identified novel angiogenic targets. From left to right, healthy eye; an eye with DR and DME shows edema in the macula, and vascular leakage, presence of microaneurysms, and cotton wool spots in the retina; an eye with PDR shows aberrant new blood vessel genesis and vascular tufts, vascular leakage, and hard exudates in the retina; an eye with ROP shows neovascular tufts in the retina; and an eye with nAMD shows inflammation and neovascularization in the choroid, and hemorrhage/vascular leakage in the macular retina. Molecular targets discussed in the text (APE-1/Ref-1, sEH, RUNX1 and FECH) are linked to the diseases in which they are implicated.

Figure 2. Mechanisms of selected angiogenic targets that underlie inflammation and angiogenesis in ocular neovascular diseases. (A) The redox function of APE1/Ref-1 regulates TFs (possibly in the nucleus) such as HIF-1α, STAT3 and NF-κB via reducing their disulfide bonds and activates inflammation and angiogenesis genes. HIF-1α regulates the expression of proangiogenic VEGF while STAT3 and NF-κB modulate the inflammatory cytokines TNF-α and IL-6. Inhibitors APX3330, APX2009, and APX2014 inhibit the redox function of APE1/Ref-1 that alters TFs from oxidized to reduced states. (B) sEH, a metabolizing enzyme of EpFAs, mediates the hydrolysis of PUFA-derived EpFAs, which enhance angiogenesis and inflammation in the eye. Bioactive EpFAs indirectly regulate the inflammatory TNF-α, IL-6, IL-1β, CCL2, ICAM-1 and angiogenic VEGF genes. sEH inhibition using a small molecule inhibitor, SH-11037, stabilizes EpFAs and improves the antiangiogenic and anti-inflammatory mechanisms.
(C) TF RUNX1 is regulated by TNF-α and high glucose via JNK, p38 and NF-κB signaling. While p38 and NF-κB directly regulate the transcription of RUNX1, p-JNK potentiates RUNX1 transcription via AP-1 activation. Ro5-3335 blocks the transcriptional activation and expression of RUNX1. (D) FECH catalyzes the insertion of ferrous iron (Fe²⁺) into PPIX to produce heme. Heme is required for the function of eNOS and complex IV of the ETC, and loss of FECH depletes HIF1-α and VEGFR2. Griseofulvin, metabolized to NMPP, and SH-17023 (4e), all inhibit ocular neovascularization by blocking FECH-directed mitochondrial dynamics with the disruption of mitochondrial morphology, ΔΨᵐ loss, decreased ATP production and complex IV dysfunction of the ETC.
Table 1. List of clinical candidate drugs primarily targeting VEGF

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of Action/Target</th>
<th>Company</th>
<th>Indication</th>
<th>Route of Administration</th>
<th>Clinical study phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSI-301 (Tarcocimab)</td>
<td>VEGF</td>
<td>Kodiak Sciences</td>
<td>nAMD, DME, retinal vein occlusion</td>
<td>Intravitreal</td>
<td>Phase 3</td>
</tr>
<tr>
<td>RGX-314</td>
<td>AAV8-VEGF</td>
<td>REGENXBIO</td>
<td>nAMD, DR</td>
<td>Suprachoroidal (Gene Therapy)</td>
<td>Phase 3</td>
</tr>
<tr>
<td>EYP-1901</td>
<td>Voloranib (TKI)</td>
<td>EyePoint</td>
<td>nAMD</td>
<td>Intravitreal</td>
<td>Phase 2</td>
</tr>
<tr>
<td>BI 764524</td>
<td>Anti-Sema3A</td>
<td>Boehringer Ingelheim</td>
<td>DR with DMI</td>
<td>Intravitreal</td>
<td>Phase 2</td>
</tr>
<tr>
<td>OTX-TKI</td>
<td>Axitinib (TKI)</td>
<td>Ocular Therapeutix</td>
<td>nAMD</td>
<td>Intravitreal implant</td>
<td>Phase 1</td>
</tr>
</tbody>
</table>

1. clinicaltrials.gov
Table 2. New drugs targeting inflammation beyond VEGF or partially involving VEGF that are undergoing clinical trials (Oral or Eyedrop)¹

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of Action/Target</th>
<th>Company</th>
<th>Indication</th>
<th>Route of Administration</th>
<th>Clinical study phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>APX3330</td>
<td>Ref-1 inhibitor (Anti-inflammatory/anti-VEGF)</td>
<td>Ocuphire</td>
<td>DR, DME, nAMD</td>
<td>Oral</td>
<td>Phase 2b</td>
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<tr>
<td>BAY1101042</td>
<td>Guanylate Cyclase activator</td>
<td>Bayer</td>
<td>NPDR</td>
<td>Oral</td>
<td>Phase 2</td>
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<tr>
<td>AKST4290</td>
<td>CCR3 Eotaxin inhibitor</td>
<td>Alkahest</td>
<td>nAMD</td>
<td>Oral</td>
<td>Phase 2</td>
</tr>
<tr>
<td>RG7774</td>
<td>CB2 receptor</td>
<td>Roche</td>
<td>NPDR</td>
<td>Oral</td>
<td>Phase 2</td>
</tr>
<tr>
<td>HCB1019 (Xiflam)</td>
<td>Connexin 43</td>
<td>InflammX</td>
<td>DR/AMD</td>
<td>Oral</td>
<td>Phase 2</td>
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<td>OPL-0401</td>
<td>ROCK 1/2 inhibitor</td>
<td>Valo</td>
<td>NPDR</td>
<td>Oral</td>
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<td>RZ402</td>
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<td>OcuTerra</td>
<td>DR</td>
<td>Eyedrop</td>
<td>Phase 1</td>
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¹clinicaltrials.gov
Figure 1