Drugs that prevent the binding of VEGF to its trans-membrane cognate receptors have revolutionized the treatment of the most important chorioretinal vascular disorders: exudative age-related macular degeneration, diabetic macular edema, and retinal vein occlusions. Pegaptanib, which binds to VEGF165 and longer isoforms, ranibizumab and bevacizumab, which bind all VEGF-A isoforms, and aflibercept, which binds VEGF-A, VEGF-B, and placental growth factor, all bind VEGF165 with high affinity. The drugs have relatively long half-lives (7 to 10 days) after intravitreal depot injections and clinical durations of action that usually exceed 4 weeks. Plasma VEGF concentrations decrease after intravitreal injections of bevacizumab and aflibercept because their systemic half-lives are extended by their Fc fragments. Extensive in vitro and in vivo testing shows that the drugs prevent VEGF-mediated activation of endothelial cells while exhibiting little evidence of toxicity. Further anti-VEGF drug development is on-going.

**KEYWORDS:** aflibercept, bevacizumab, pegaptanib, pharmacokinetics, ranibizumab, VEGF

Choroidal and retinal vascular diseases such as exudative age-related macular degeneration (AMD), diabetic macular edema (DME) and retinal vein occlusions are among the leading causes of blindness in industrialized nations \[1,2\]. For decades, focal and grid laser photocoagulation of the macula were the only treatments of proven benefit for these disorders, but despite timely and appropriate application of laser, significant improvements in vision were unusual and loss of vision was frequent. Reasons for vision loss include breakdown of the blood–retinal barrier with the subsequent accumulation of macular edema and the pathologic growth of neovascular tissue, features that are common to angiogenesis. More effective treatments of these conditions emerged only after researchers acquired a detailed understanding of the underlying pathophysiology.

Recognizing that VEGF plays a critical, rate-limiting role in angiogenesis \[3\], developers created VEGF-binding drugs that prevent activation of the cognate receptors. Four drugs – pegaptanib (Macugen®, Eyetech, New York, NY, USA), ranibizumab (Lucentis®, Genentech, S. San Francisco, CA, USA/Roche, Basel, Switzerland; distributed outside of North America by Novartis), bevacizumab (Avastin®, S. San Francisco, CA, USA/Roche, Basel, Switzerland) and aflibercept (Eylea®, Regeneron, Tarrytown, NY, USA; distributed outside of the United States by Bayer Healthcare) – are currently used to treat ocular angiogenesis. This manuscript aims to describe the biochemical rationale that led to the development of these drugs, detail their pharmacokinetic and pharmacodynamic properties and discuss the pre-clinical studies that support their use in humans.

**Methods (literature search)**
Medline searches using the keywords aflibercept, bevacizumab, pegaptanib and ranibizumab were performed to identify manuscripts.
Additional manuscripts were identified from the reference lists of these articles.

Overview
Angiogenesis, the physiologic growth of new blood vessels from pre-existing vasculature, is essential to embryogenesis and is an integral component of wound healing and reproductive function in adults, whereas neovascularization is the pathological growth of vessels that occurs in a variety of disorders such as solid tumors, rheumatoid arthritis and AMD. Angiogenesis requires the coordination of multiple biochemical events, which include the sequential binding of ligands to their transmembrane receptors. Since VEGF binding to its cognate receptors VEGF receptor (VEGFR) 1 and VEGFR2 is a rate-limiting step in vascular growth, VEGF has become an important target for the inhibition of angiogenesis.

VEGF is actually several molecules that segregate into seven closely related families: VEGF-A, VEGF-B, VEGF-C, VEGF-D, the onf virus expressed VEGF-E, snake venom VEGF-F and placental growth factor. Variable splicing of the VEGF-A gene products creates six major isoforms (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206), eight minor isoforms and the fibrin split product VEGF110 but VEGF165 (MW of the dimer: 38.2 kDa) appears to be the primary mediator of ocular angiogenesis. VEGF165 synthesis is upregulated by hypoxia, nitric oxide, growth factors (basic fibroblastic growth factor, epidermal growth factor, insulin-like growth factor, keratinocyte growth factor and platelet-derived growth factor), inflammatory mediators (IL-1, IL-6, IL-10, TNF-β and prostaglandin E2) and mechanical forces such as shear and stretch [4].

Several lines of evidence link VEGF with the development of important chorioretinal vascular conditions. Exudative AMD, diabetic retinopathy and retinal vein occlusions are associated with elevated intraocular concentrations of VEGF [5]. VEGF165, VEGF-B and placental growth factor have been detected in excised choroidal neovascular membranes (CNVMs). Intravitreal placental growth factor levels are elevated in patients with proliferative diabetic retinopathy (PDR) [16]. The intraocular injection of exogenous VEGF produces retinal findings similar to those in diabetic retinopathy: microaneurysms, hemorrhages and neovascularization of the retina and iris [7].

VEGF promotes proliferation, migration and survival of vascular endothelial cells, increases vascular permeability, dilates blood vessels and attracts endothelial cell precursors and monocytes. Within minutes to hours after exposure to VEGF, activated vascular endothelial cells synthesize proteins such as matrix metalloproteinases. Both VEGF (at embryonic day 9) and VEGFR2 (at post-natal day 6.5) have been found in mouse retinal pigment epithelium (RPE) to support chorioidal development. In vitro VEGF neutralization increases RPE apoptosis while systemic neutralization separates RPE from photoreceptors and decreases fenestrations in choriocapillaris. Though vascular endothelial cells and, to a lesser degree, RPE, Müller and neurosensory retinal cells are the primary targets of VEGF, all retinal cells, Müller cells, pericytes, limbal blood vessels and corneal epithelial, stromal and endothelial cells can synthesize VEGF [8].

Drug development
Orally administered receptor tyrosine kinase inhibitors have been used to treat malignancies since 1988. Many of these drugs prevent VEGF activation, but their binding is non-specific since platelet-derived growth factor, epidermal growth factor and fibroblast growth factor receptors (among others) are also affected. Unfortunately, their small molecular weights (~0.4 kDa) make receptor tyrosine kinases inappropriate for depot intravitreal injections and long-term oral administration leads to adverse systemic events. Drugs described in this manuscript, however, specifically neutralize soluble VEGF dimers, inhibit dimerization of the trans-membrane receptors VEGFR1 and VEGFR2 and prevent activation of vascular endothelial cells. Two recombinant strategies were employed by early anti-VEGF drug developers: creation of a VEGF165-specific aptamer (pegaptanib) and synthesis of a pan-VEGF-A-binding antibody (monoclonal antibody [Mab], bevacizumab) and antibody fragment (Fab, ranibizumab).

Some developers believed that binding only VEGF165 would adequately reduce VEGF signaling and avoid adverse events associated with pan-VEGF-A suppression, so they created pegaptanib, a 50-kDa pegylated aptamer that attaches to the heparin-binding site of longer VEGF-A isoforms with high affinity (KD = 50 pM) [9]. Since the naturally occurring isoforms VEGF183, VEGF189 and VEGF206 are bound to intercellular matrix and trans-membrane proteoglycans, pegaptanib is said to be VEGF165 specific. Pegaptanib cannot bind the shorter naturally occurring isoforms VEGF121 and VEGF145, or the fibrin split product VEGF110 [10].

Pegylation refers to the modification of a biologically active molecule by covalently linking it to polyethylene glycol to improve its pharmacological behavior, increase its pharmacokinetic half-life, reduce the propensity to metabolism by endonucleases and decrease renal excretion. Conjugation of the 28-base aptanib with the 40-kDa polyethylene glycol moiety represents the first application of pegylation to oligonucleotides [11].

Early pharmacokinetic studies in rhesus monkeys determined that an anti-VEGF Fab and a Mab against epidermal growth factor had intravitreal half-lives of 3.2 and 5.6 days, respectively [12]. Whereas the Fab rapidly penetrated the retina, the Mab stopped at the inner retina. This observation perpetuated the prevailing belief that the inner plexiform layer prevented penetration of molecules larger than 76 kDa [13]. Therefore, bevacizumab was not developed for ocular conditions, and the first ophthalmic trial used intravenous bevacizumab [14]. However, full penetration of the retina was recognized when patients with exudative AMD [15] and edema due to a central retinal vein occlusion [16] responded successfully to intravitreal bevacizumab.

Bevacizumab is a humanized Mab that attaches to the VEGF-binding domain (amino acids 81 through 92) of all VEGF-A isoforms. The murine anti-VEGF antibody Mab
A.4.6.1 was humanized and reconstructed with Fab variants to create the full-length bevacizumab antibody with a high binding affinity for VEGF_{165} (K_D = 58–1100 pM) [17,18]. Bevacizumab is synthesized in Chinese hamster ovary cells as a 149-kDa antibody composed of two 453-amino acid heavy chains and two 214-amino acid light chains. Bevacizumab is packaged in 100 mg (4 ml) and 400 mg (16 ml) single-use vials and should be stored between 2 and 8°C. The manufacturer recommends that the vials should be protected from light but not be shaken or frozen. One week after fractionation and storage at 2–8°C, a 1.6% loss of bevacizumab activity was noted, and 6 months later, the degradation of chilled (4°C) and frozen (-10°C) bevacizumab was 15.9 and 12%, respectively [19]. Another study, however, showed a greater loss of activity (-20%) after freezing at -20°C for 5 days [20].

Intraocular doses of bevacizumab are drawn without dilution from the manufacturer supplied vial. Though some ophthalmologists remove single doses and discard the remainder of the vial, most physicians acquire individually fractionated doses (1.25 mg/0.05 ml) in plastic syringes from compounding pharmacies. Despite rigorously followed compounding procedures, significant differences in bevacizumab concentrations in these fractionated syringes have been reported.

Micron-sized bevacizumab macroaggregates have been found in syringes and since antibody aggregates may decrease efficacy and increase immunogenicity, concerns regarding their effects have been raised [21]. Macroaggregates have been attributed to contamination with silicone oil and repackaging from the manufacturer’s container [22]. An in vitro model showed that a protein–protein interactive hotspot may spontaneously form dimers that can be prevented with dexamethasone and betamethasone, but not triamcinolone. Since elevated intraocular pressure plagues 9.9% of patients receiving intravitreal bevacizumab but only 3.1% of those receiving ranibizumab, some physicians suggest that macroaggregates are to blame [23].

Ranibizumab was developed from a different murine monoclonal VEGF-binding antibody (MB1.6 antibody variant) from bevacizumab. After the substitution of 5 amino acids in the clonal VEGF-binding antibody (MB1.6 antibody variant) from [23]. The simplified cartoon in Figure 1 compares the VEGF-binding mechanisms used by the four anti-VEGF drugs. Table 1 lists several important biochemical and pharmacokinetic properties of the four drugs.

**Pharmacokinetics**

The four VEGF-binding drugs have been tested in animals and humans, but complete data, particularly pharmacokinetic values in humans, have not been obtained. Some pharmacokinetic characteristics, however, appear common to each of the drugs. Since the vitreous has a gel-like consistency composed of aligned collagen and glycosaminoglycans, it represents a barrier to rapid drug distribution. Following intravitreal injections, each of the anti-VEGF drugs leaves the eye according to a first-order decay profile. Most investigators use a one-compartment model to describe intraocular drug behavior though some use a two-compartment model to account for a rapid initial drug distribution. Drugs exit the eye by crossing the retina and RPE to the choroidal circulation, passing through the ciliary body and iris or moving into the anterior chamber by diffusion and bulk flow before exiting through the trabecular meshwork. None of the drugs appears to undergo degradation within the eye. Systemic half-lives vary greatly (from hours to weeks) before drug elimination via glomerular filtration or pinocytotic elimination.

Pegaptanib has an intravitreal half-life of 3.9 days in monkeys [11]. Though its half-life in human eyes has not been determined, its estimated half-life (7 days) combined with an excellent VEGF_{165}-binding affinity are consistent with 6-week dosing. After entering the systemic circulation, the maximum serum concentration is reached in 1–4 days and the serum half-life is 10 days. Pegaptanib is metabolized by endo- and exonucleases and is excreted primarily in the urine, though dose adjustments are not necessary for patients with renal impairment. Pegaptanib appears to withstand conformational changes since drug sampling 7 and 28 days after injection showed fully functional molecules.

The intravitreal, aqueous and serum half-lives of bevacizumab in rabbits are 4.32, 4.88 and 6.8 days [28,29], though other investigators measured longer intraocular durations 6.61, 6.51 and
5.87 days [30]. Peak concentrations are achieved in the aqueous, serum and contralateral aqueous at 3, 8 and 8 days. In the injected eyes, the maximum aqueous concentration is 9.4% of that achieved in the vitreous whereas in the fellow eyes, the maximum aqueous concentration is twice that in the vitreous (1.6125 vs 0.335 ng/ml) [30]. Bevacizumab concentrations in the retinas of fellow eyes remained above the IC$_{50}$ for VEGF for up to 8 weeks [31]. A pharmacokinetic model suggested that 96% of drug clearance from the vitreous was through the retina and uvea, rather than via the anterior chamber [28].

After intravitreal injections into rabbits, bevacizumab appeared in the subretinal space within 2 h [32], the inner retina and choroid within the first day and the outer layers and choroid in subsequent days, but no drug was found at 4 weeks [33]. Peak drug concentrations within the retina were twice those in the vitreous, with most localized to blood vessel walls. Immortalized bovine retinal endothelial cells internalize bevacizumab though in vitro barrier function does not appear to be adversely affected [34]. Stained Müller cells suggest that active transport mechanisms may be responsible for moving bevacizumab through the retina [35]. In vitro porcine RPE cells internalized and concentrated bevacizumab for up to 3 months, whereas ranibizumab was found by 1 h but not at 1 day. Neither drug caused RPE toxicity or decreased VEGF secretion, but since binding to Fc receptors can activate complement and lead to cell death, concerns over possible toxicity linger [36]. Since bevacizumab, but not ranibizumab, contains sugar moieties, bevacizumab uptake by RPE might be via mannose receptors. Laser photocoagulation upregulates Fc receptors to more rapidly distribute IgGs to the systemic circulation [37].

The intravitreal half-life of bevacizumab in monkeys is 3.1 days with rapid drug penetration through the retina. After intravitreal injections, bevacizumab concentrations peak in the aqueous after 1 day, in the fellow eye at 3 days and in serum after 1 week. Serum VEGF concentrations decreased for 4 weeks but aqueous VEGF in the fellow eye was unaffected.

In human eyes with endophthalmitis and retinal detachment, the half-life of bevacizumab was estimated to be 3 days [38]. In non-inflamed eyes, bevacizumab was found to have a half-life of 6.7 days with a two-compartment model [39] and from 8.2 to 10 days in a one-compartment model [40–42]. Based on the elimination data, the authors estimated that intravitreal
bevacizumab concentrations were sufficient to warrant treatment intervals of 6–7 weeks [38]. The half-life of bevacizumab in human serum is 17–21 days, similar to that of other full-length antibodies. From 1 to 28 days after intravitreal bevacizumab injections, no unbound bevacizumab was found in the aqueous of the fellow eyes [43].

Topical delivery of bevacizumab is unlikely to produce therapeutic intraocular concentrations because of rapid elimination in tears and poor penetration of molecules larger than 5 kDa through intact epithelium. Topical and subconjunctival bevacizumab can penetrate the corneal stroma in the presence of neovascularization [44]. Topically applied bevacizumab only minimally enters the anterior chamber, but after subconjunctival injections, bevacizumab is detected in the anterior chamber at 12 h with peak concentrations at 36 h. Aqueous exposure is 2000-fold less than that after intravitreal injections and aqueous concentrations are less than those in the serum but higher than those in the aqueous of the fellow eye. Serum levels after subconjunctival injections are comparable to those following intravitreal injections [45].

Neovascularization due to PDR may respond to bevacizumab doses as low as 6.25 μg [46] and regression of vessels in the contralateral eye has been reported after an intravitreal injection. In a retrospective study of 55 eyes, there was a greater improvement in the central foveal thickness of fellow eyes with DME after bevacizumab injections (417–372 μm) than after ranibizumab injections (399–407 μm) at 4 weeks, though no significant changes in VA occurred in either group [47].

Most studies suggest that vitrectomy decreases the half-life of intravitreal molecules. The intravitreal half-life of VEGF was reduced from 2.46 h to 12.5 min in vitrectomized eyes [48]. When lensectomy and vitrectomy were performed in monkeys, postoperative VEGF levels were significantly reduced and the postoperative half-life of bevacizumab was 1.5 days [49]. In previously vitrectomized eyes with DME, intravitreal injections of bevacizumab produced poor clinical responses [50]. The intra-vitreal half-life of bevacizumab in rabbits decreased from 4.22 to 2.08 days after lensectomy and vitrectomy [51]. However, after lens-sparing vitrectomies in rabbits, intravitreal bevacizumab concentrations followed a two-phase distribution, in which the first phase but not the second was more rapid in vitrectomized eyes. The authors concluded that the overall half-lives of intravitreal bevacizumab are not substantially different in vitrectomized (6.99 days) and non-vitrectomized (7.06 days) eyes. They acknowledged that the large lens and inability to completely remove vitreous from the surface of the retina may have influenced their results [52].

In rabbit eyes filled with silicone oil, the bevacizumab fluid bubble disappeared from the oil phase by 48 h. Bevacizumab concentrations peaked at day 7 in the iris/ciliary body and serum and day 14 in the aqueous, retina and choroid, with maximum and minimum exposure to bevacizumab seen in the choroid and aqueous, respectively. Terminal half-lives of bevacizumab were 3.5 days in oil, 4.5 days in aqueous, 7.98 days in plasma and 3–5 days in ocular tissues, while the mean residence times were 10–13 days. The terminal half-lives appeared to be unaffected by oil, but peak bevacizumab concentrations and exposures were lower than in non-vitrectomized eyes. Bevacizumab levels in solid tissues of fellow eyes were similar to those in serum [53].

After intravitreal injection into rabbits, ranibizumab has an intravitreal half-life of 2.6–2.88 days [51,54,55] and the aqueous concentration peaks at 3 days. The maximum concentration in the aqueous is 11% that of the vitreous and total drug exposure in the aqueous is 14% that of the vitreous. Ranibizumab fully penetrates the retina by 1 day after injection. Drug concentrations in the serum are either very low (1/10,000 that of the vitreous) [54] or undetectable [55].

| Table 1. Major structural and pharmacokinetic characteristics of the four available anti-VEGF drugs. |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Structure                        | Pegaptanib      | Ranibizumab     | Bevacizumab     | Aflibercept     |
| Molecular weight (kDa)           | 50              | 48              | 149             | 115             |
| Production assay                 | Chemical synthesis | Escherichia coli | Chinese hamster ovary | Chinese hamster ovary |
| K<sub>0</sub> for VEGF<sub>165</sub> (pM) | 50               | 46–192          | 58–1100         | 0.5             |
| Binding targets                  | VEGF<sub>165</sub> | VEGF-A          | VEGF-A          | VEGF-A          |
|                                  |                  |                  | VEGF-B PGF      |                 |
| Dose (volume)                    | 0.3 mg (0.9 ml) | 0.5 mg (0.05 ml) | 1.25 mg (0.05 ml) | 2 mg (0.05 ml)  |
| Intravitreal half-life (days)    | 3.9 (monkeys)   | 2.6–2.88 (rabbits) | 4.32–6.61 (rabbits) | 4.5–4.7 (rabbits) |
|                                  |                  | 3–3.2 (monkeys) | 3.1 (monkeys)   |                 |
|                                  |                  | 7.1 (humans)    | 6.7–10 (humans) |                 |
| Serum half-life humans (days)    | 10              | 0.25            | 21              | 18              |
In a rabbit model of retinal neovascularization, injections of small quantities of ranibizumab every third day were as effective as one 0.5 mg injection. The authors suggest that this regimen mimics dosing from an extended-duration drug delivery device [56].

The half-lives of ranibizumab and a related Fab in monkeys are 3 and 3.2 days [12,57] and serum concentrations are 1000-fold lower than those in the vitreous. Intravitreal ranibizumab distributes rapidly to the retina within 6–24 h [57] and therapeutic concentrations are maintained for 1 month [57].

The intravitreal half-life of ranibizumab in humans has been estimated as 4.8–9 days according to mathematical and population models, but in a prospective sampling study, the aqueous half-life was 7.1 days. Serum concentrations of ranibizumab are estimated to be 90,000-fold lower than intraocular concentrations. Contralateral effects after intravitreal ranibizumab have not been noted in animal models or clinical reports [58], and the ANCHOR and MARINA trials did not show decreased CNVM development in fellow eyes [59].

Aflibercept has a half-life of 4.7 days in rabbits [60], and though intravitreal pharmacokinetic studies have not been performed in humans, the intermediate size of the molecule – between ranibizumab and bevacizumab – suggests an intravitreal half-life of 9 days. The half-lives of unbound and bound aflibercept in human serum are 1–3 days and 18 days, respectively.

Reflux of intraocular fluid into the subconjunctival space has been observed after 31% of intravitreal injections, with a higher incidence in patients over the age of 65 years [61]. In rabbits, subconjunctival blebs formed after injections through 27- and 30-gauge (but not 32-gauge) needles [62] but when the intraocular pressure was lowered before the injection with an anterior chamber paracentesis, a small bleb was noted following injections only through a 27-gauge needle. Oblique insertion of the needle through the sclera reduces the size of the bleb by 50% [63]. In a retrospective review of 234 bevacizumab (0.1 ml) injections, post-injection paracenteses for elevated intraocular pressure were performed after 26 and 38% of injections through 27- and 30-gauge needles, respectively (p = 0.05). The semi-quantitative grades for volume of reflux were 2 and 1, respectively (scale of 0–4) [64]. The amount of reflux appears to be reproducible as 76–85% of eyes behave the same with subsequent injections.

The relationship between volume of reflux and drug efficacy and safety remains unclear. In a retrospective study with bevacizumab and ranibizumab injections, the amount of reflux did not correlate with foveal thickness [65]. Studies that used fluorescein [66] or methylene blue-stained drug [63] noted reflux of dye-containing liquid into the subconjunctival space, suggesting that some of the efflux contains active drug.

**Pharmacodynamics**

The drugs discussed in this review bind exclusively to diffusible VEGF, but the resultant clinical responses are complex, disease dependent and may occur outside the eye. Adverse events are relatively common with intravitreal injections, though events that cause severe injury or death are unusual. The systemic administration of VEGF-binding drugs in oncologic and pilot AMD trials was accompanied by increased incidences of hypertension, proteinuria and thromboembolic events, particularly myocardial infarction and stroke. It remains unclear, however, whether the same adverse events are caused by the intravitreal administration of these drugs. Phase III trials that compared VEGF-binding drugs with observation or non-VEGF–binding treatments failed to identify elevated risks of systemic adverse events (SAEs). Meta-analyses, however, suggest that intravitreal therapy may increase the risks of significant SAEs. As a precautionary measure, all prospective anti-VEGF drug trials exclude patients that have recently suffered systemic thromboembolic events. Despite the large body of accumulated data, the risk of SAEs due to intravitreal therapy remains an important unresolved issue. Whereas most of these events fall within the broad framework of pharmacodynamics, the complexity of the issues and limitations of space make a detailed discussion beyond the scope of this manuscript.

Randomized clinical trials show that pan-VEGF-binding drugs (bevacizumab, ranibizumab and aflibercept) are superior to VEGF165-specific binding (pegaptanib) for the treatment of AMD, with smaller differences among DME patients. Though these differences have often been attributed to the limitations of VEGF165-specific binding, the importance of other factors such as molecular binding sites (heparin vs receptor) and pharmacokinetics cannot be excluded.

However, the effects of anti-VEGF drug injections on the concentrations of intraocular and systemic growth factors, cytokines and chemokines are important to this discussion. Compared to age-matched controls, patients with exudative AMD have similar serum concentrations of VEGF. Twenty-eight days after intravitreal injections of bevacizumab but not ranibizumab, there was a 42% reduction in plasma VEGF levels [67]. Compared to controls and eyes with types 1 and 2 CNVM, aqueous VEGF levels are significantly higher in eyes with type 3 CNVM and remain reduced through 2 months after intravitreal bevacizumab [68]. In eyes with neovascular AMD and myopic CNVM that received intravitreal bevacizumab injections, aqueous VEGF concentrations decreased from 102 to 18 pg/ml and 20 to 4 pg/ml respectively, whereas pigment epithelium-derived factor (PEDF) levels increased from 11 to 38 pg/ml and 20 to 126 pg/ml, respectively [69]. In eyes with neovascular AMD, aqueous VEGF levels were significantly higher in treatment naïve and recurrent neovascularization groups than in those with regressed CNVM, though VEGF levels did not differ significantly from controls. IL-6 and IL-8 levels correlated with the size of the active lesions [70]. One day after intravitreal injections of bevacizumab, VEGF levels decreased, but IL-6 levels were elevated from days 1 through 7. Levels of IL-8 and TGF-β were elevated only at day 7, suggesting that they were due to IL-6. The authors also postulated that IL-8 and TGF-β could stimulate contraction of CNVM [71].

After monthly injections of ranibizumab to patients with exudative AMD, aqueous VEGF levels fell from 94 pg/ml to
less than the detectable limit in eight of nine patients and ranibizumab concentrations were 71 and 96 ng/ml after the first and second injections. In patients receiving bimonthly injections, average VEGF levels at 2 months fell from 153 to 69 pg/ml and were less than the detectable limit in only 2 of 17 patients. Ranibizumab concentrations in 15 of 17 patients averaged 2.5 ng/ml and were below the minimal detectable limit in 2 of 17 patients. Because of the relatively high VEGF concentrations at 2 months, the authors hypothesized that ranibizumab suppresses VEGF for 1 month but not 2 months and that the minimal ranibizumab concentration able to suppress VEGF was 5 ng/ml, which corresponds to a vitreous concentration of 11–16.5 ng/ml [72]. This is consistent with a previous report that half the maximal inhibitory concentration of ranibizumab needed to decrease VEGF was 11–27 ng/ml.

Significantly increased aqueous concentrations of monocye chemotactic protein (MCP)-1, IL-6, IL-8, apelin and VEGF were measured in eyes with background diabetic retinopathy, but after intravitreal bevacizumab, only VEGF decreased to subnormal levels [73,74]. In a series of patients with AMD and DME, serum concentrations of VEGF were significantly reduced 1 week and 1 month after intravitreal injections of bevacizumab but not after ranibizumab and pegaptanib injections [75].

In eyes with PDR, intravitreal bevacizumab lowered aqueous VEGF levels at 1 week from 302 pg/ml to less than 31 pg/ml (lower limit detectable by ELISA) but did not affect levels in the fellow eyes [76]. In patients with neovascular glaucoma or PDR who received intravitreal bevacizumab injections, aqueous VEGF concentrations fell from 676 to 7 pg/ml by 7 days and stayed below baseline through 8 weeks, whereas PEDF levels remained unchanged [77]. The same authors reported a reduction in average serum VEGF levels from 117 to 9.7 pg/ml at 1 day and 25.9 pg/ml at 1 month after bevacizumab [78]. In another study of patients with PDR, aqueous levels of VEGF, IL-1, IL-5, IL-10, IL-12, IL-13 and IFN-γ were decreased after bevacizumab injections [79].

Compared to controls, aqueous concentrations of IL-6, IL-8, IL-17 and VEGF are elevated in eyes with retinal vein occlusion. In eyes that received intravitreal triamcinolone, the concentrations of IL-6, IL-17, IP-10, MCP-1, PDGF and VEGF were significantly reduced, whereas bevacizumab reduced only VEGF. The post-injection concentration of VEGF was lower in the bevacizumab group compared to the triamcinolone group (p = 0.06) [80,81].

In babies with threshold retinopathy of prematurity, serum VEGF levels are reduced for up to 2 weeks after the intravitreal injection of bevacizumab [82].

After intravitreal injections of ranibizumab, aqueous VEGF levels are decreased from 85.57 pg/ml to below physiologic levels of controls (p = 0.001) [83,84]. In large randomized, controlled trials, serum VEGF levels are diminished after intravitreal injections of bevacizumab but do not appear affected by intravitreal ranibizumab [85,86]. Intravitreal aflibercept decreases serum VEGF to levels similar to those resulting from bevacizumab.

Bevacizumab decreases the concentration of VEGF-A in human breast milk [87] whereas ranibizumab does not.

Various cancer studies have reported artificially high serum VEGF levels because the blood extraction process activates platelets and releases VEGF [88]. Blood should be collected in tubes with anticoagulants such as ethylenediaminetetraacetic acid [75,77], though some manuscripts fail to report their methodology [67,82].

Pre-clinical studies
In vitro studies
Several studies have quantitatively compared drug inhibition of VEGF-mediated processes. Bevacizumab was equally effective to Mab A.4.6.1 at inhibiting in vitro proliferation of human umbilical vein endothelial cells (HUVECs) and in vivo growth of tumors. Ranibizumab had a 22-fold increase over Fab-12 in VEGF-binding affinity assays and a 12- to 140-fold increase over Fab-12 in kinetic competition assays. Compared to bevacizumab, ranibizumab inhibited VEGF-induced mitogenesis by 30–100-fold [36], VEGF-mediated proliferation of vascular endothelial cells in a perfused organ culture by sixfold (pegaptanib was ineffective) [89] and increased VEGF binding by 5–20-fold [24].

Ranibizumab and VEGF Trapb182 equally inhibited in vitro MAP kinase activation of HUVECs and VEGF-induced proliferation and migration, whereas bevacizumab was 10-fold less potent [90]. In a related study, aflibercept was 10–129 times as effective as ranibizumab and bevacizumab at inhibiting VEGF-mediated vascular endothelial cell migration and calcium mobilization. These authors speculated that the 1000-fold difference in VEGF reagent concentrations was responsible for the disparate results [15]. Although both studies tested the effects of anti-VEGF drugs on HUVECs, these cells may not respond like retinal vascular endothelial cells. Furthermore, microvascular endothelial cells from different species often behave differently, though the response of bovine retinal endothelial cells is similar to human retinal vascular endothelial cells. Readers must, therefore, note the origin of the vascular endothelial cells when critically evaluating in vitro studies [91–94].

The effects of anti-VEGF drugs on several species of vascular endothelial cells – rat, pig, cow, monkey and human – have been studied in vitro. Bevacizumab, ranibizumab and pegaptanib reduced VEGFR2 expression and VEGF synthesis without affecting cell viability [95], but bevacizumab and ranibizumab suppressed cell proliferation and augmented apoptosis whereas pegaptanib did not [96]. Bevacizumab and ranibizumab equally decreased cellular adhesion to fibronectin, cellular proliferation and migration, matrigel-induced tubule formation and vascular assembly, VEGF secretion and VEGFR expression [97,98]. Ranibizumab (44.1%), bevacizumab (38.2%) and pegaptanib (35.1%) reduced choroidal endothelial cell proliferation in a dose-dependent manner and bevacizumab and pegaptanib (but not ranibizumab) mildly decreased ARPE-19 proliferation [99].
The integrity of the blood–retinal barrier is determined primarily by the retinal vascular endothelial cells and their tight junctions. VEGF165, but not placental growth factor, decreases transendothelial electrical resistance and downregulates claudin-1 expression by immortalized bovine vascular endothelial cells [100]. Ranibizumab and aflibercept were equally effective at restoring claudin-1 expression by bovine endothelial cells [101]. VEGF121 and VEGF165 were found to be equally potent at stimulating endothelial cell proliferation, which was equally prevented by bevacizumab and ranibizumab, whereas pegaptanib prevented only VEGF165-mediated proliferation. Bevacizumab significantly reduced the number of fenestrations in rat choriocapillaris endothelial cells from 69 to 53 per μm² at day 7 [102]. Bevacizumab inhibits the growth of choroidal endothelial cells by halting the cell cycle at the G0/G1 stage [103].

Vitreo may alter the results of suppression studies as co-culturing human retinal vascular endothelial cells with hyalocytes decreases the effects of IL-1, IL-6, TNF-α, VEGF, bevacizumab, dexamethasone and fenofibrate on the endothelial cells [104].

Bevacizumab and ranibizumab reduce the proliferation of RPE cells by arresting division at the G1/S phase [105] and RPE barrier function [106] with bevacizumab having a more profound and longer lasting effect, but only bevacizumab reduces RPE phagocytosis by over 50%. VEGF suppression did not impair RPE wound healing [107]. However, another study showed that bevacizumab and ranibizumab had minimal effect on RPE barrier function as measured by trans-epithelial resistance; K⁺, Na⁺ and Cl⁻ flux and claudin synthesis [108]. Bevacizumab is non-toxic to RPE cells [95].

Afibercept did not cause significant changes in morphology, apoptosis, viability or proliferation of human scleral fibroblasts [109], trabecular meshwork cells [109], ARPE-19 [109,110], retinal ganglion (RGC)-5 cells and 661W cells and induced fewer changes than were seen with bevacizumab and ranibizumab [110].

Bevacizumab is not toxic to RGCs [95,111,112] at doses that inhibit choroidal vascular endothelial cell proliferation [113], but it stimulates RGC proliferation [114]. After treatment of retinal neurons with ranibizumab and bevacizumab, VEGF expression ceases and does not return for 14 days. No electroretinogram changes were noted when isolated bovine retinas were perfused with pegaptanib [115].

Bevacizumab is non-toxic to corneal epithelial cells, but different studies have shown it to be safe [116] or toxic [117] to endothelial cells at concentrations used in clinical practice. Bevacizumab reduces corneal wound healing and limits integrin expression [118].

Topical doses of bevacizumab that were incubated with pooled plasma significantly increased in vitro coagulation by decreasing the prothrombin time [119].

Bevacizumab injections are non-toxic to the RGCs and nerve fibers of the developing rabbit retina [122], but they interfere with blood vessel development [122]. Bevacizumab decreases programmed retinal cell death (apoptosis) and increases proliferation (gliosis) and reactivity [124] in juvenile rabbit eyes. After both single and repeated injections of bevacizumab into adult rabbit eyes, ERGs and histological analyses were normal up to 4 weeks later [33,125,126]. However, bevacizumab disrupts photoreceptor mitochondria and apoptotic protein (bax, caspase-3, caspase-9) expression [127] and causes mild dose-related apoptosis [128,129]. Single injections of bevacizumab do not cause structural changes, gliosis or apoptosis in the porcine retina, but VEGF transcription is downregulated in the retina (by 60%) and RPE (by 35%) [130].

Early injection of bevacizumab prevents VEGF-induced disc edema, vascular hyperemia and tortuosity and iris neovascularization, but late injection leads to capillary closure [131]. After injections of bevacizumab into streptozotocin-induced rats, concentrations of VEGF decreased for 1 month and CD34 decreased for 2 months, suggesting that VEGF suppression occurred quickly but the effects on endothelial cell proliferation were delayed [132].

Neurotrophic growth factor and VEGF are upregulated after debridement of the corneal epithelium, but bevacizumab drops decrease both, therefore suggesting that a paracrine loop exists between the two compounds [118]. Corneal neovascular growth was limited by bevacizumab (41%) and pegaptanib (18%). After suture removal from rabbit corneas, subconjunctival sunitinib decreases neovascularization more than bevacizumab (1.6- vs 1.4-fold) and topical administration is more effective than subconjunctival injections (2.5- vs 2.3-fold). Compared to the full-length antibody, the small-molecular-weight sunitinib penetrates the cornea better when applied topically [133].

In rats with endothoxin-induced uveitis, intravitreal bevacizumab increases the concentrations of MCP-1, RANTES and IFN-γ, therefore suggesting that bevacizumab should be used with caution in patients with uveitis [134].

Ranibizumab, pegaptanib and bevacizumab did not diminish laser-induced CNVM in rats [135], but bevacizumab significantly suppressed CNVM growth better than ranibizumab in transgenic mice [136].

Rat RGCs exhibited no toxicity after exposure to bevacizumab and ranibizumab, though mitochondrial swelling occurred after high doses of pegaptanib [137]. In rabbit eyes injected with three times the therapeutic doses of bevacizumab, ranibizumab and pegaptanib, decreased b-wave amplitudes were noted on ERGs at 8 weeks and in eyes treated with bevacizumab and ranibizumab, but not pegaptanib, reduced protein kinase-C labeling in rod bipolar cells was noted [138].

After injuries to the cornea with cautery and alkali, bevacizumab was generally better than ranibizumab and pegaptanib at reducing areas of neovascularization [139,140].

In a matrigel-induced CNVM model in rats, aflibercept injections at 2–6 days prevented CNVM development and at 10 days prevented progression and induced regression.
Aflibercept also decreased total lesion volume and prevented further leukocyte infiltration and fibrosis [141]. Subcutaneous and intravitreal aflibercept prevents laser-induced CNVM, and blood-retinal barrier breakdown and CNVM development in transgenic mice that overproduce VEGF from the photoreceptors [142]. Intravenous and intravitreal aflibercept nearly completely prevents the development of grade 4 CNVM in cynomolgus monkeys [143]. Systemic aflibercept prevents corneal vascularization in mouse eyes with high-risk grafts and corneal injuries [144,145]. Aflibercept prevented pathologic neovascularization in a canine model of retinopathy of prematurity, but high doses also prevented retinal revascularization. The authors suggest that correct drug dosing is critical for the successful treatment of ROP [146].

Conclusion
Numerous in vitro and in vivo models have shown that VEGF-neutralizing drugs are generally safe and effective. Though the drugs have different VEGF-binding affinities and pharmacokinetic profiles that may determine clinical efficacy and side effects in some patients, randomized clinical trials suggest that most treatment naïve patients respond similarly regardless of which pan-VEGF-A binding drug is used.

Expert commentary & five-year view
Pan-VEGF-A binding with bevacizumab, ranibizumab and aflibercept effectively treats many patients with exudative AMD, DME and retinal vein occlusions. Though VEGF₁₆₅-specific binding by pegaptanib was an exciting approach to exudative AMD, most physicians have abandoned its use because of poor results. Pegaptanib possesses excellent pharmacokinetics and a high VEGF-binding affinity, but its inability to bind VEGF₂₁ and the fibrin split product VEGF₁₁₀, or its attachment to the heparin-binding site (not the VEGF receptor-binding site), may account for its clinical performance. The importance of binding VEGF-B and placental growth factor by aflibercept is not yet known. Whether this accounts for its ability to flatten treatment-resistant RPE detachments and dry persistent edema and subretinal fluid remains to be determined.

Serum VEGF concentrations decrease after intravitreal injections of bevacizumab and aflibercept (but not ranibizumab) probably because the Fc fragment extends their serum half-lives. Concerns over vascular occlusive events due to VEGF suppression have been raised, but large prospective trials have failed to consistently identify problems.

Pegaptanib, ranibizumab and aflibercept have all been approved by the US FDA for the treatment of exudative AMD, but the use of bevacizumab remains off-label. Compounding of bevacizumab results in very low unit costs (US $50–US$75/dose), which is one reason that bevacizumab is the most frequently used ophthalmic anti-VEGF drug. Low acquisition costs of bevacizumab have probably helped create the considerable body of pre-clinical data, which is greater than that of the other anti-VEGF drugs.

Vitrectomy has become a popular treatment for conditions such as vitreomacular traction and epiretinal membrane, and many eyes with DME undergo vitrectomy with internal limiting membrane removal. Most pharmacokinetic studies show that vitrectomy shortens intravitreal drug half-lives and probably decreases the effectiveness of intravitreal injections, so for patients that may require anti-VEGF therapy the risks of vitrectomy should be very carefully considered.

Two new VEGF-binding drugs – DARPin MP0112 and ESBA 1008 – are entering Phase III trials for exudative AMD. Our current drugs maximally inhibit VEGF, so some investigators believe that new agents can only differentiate themselves by providing an extended duration of action. In a four-patient DME trial, DARPin MP0112 appeared to have an intravitreal half-life of 13 days, but in a subsequent AMD trial its duration of action was comparable to ranibizumab.

Other pro-angiogenesis molecules such as PDGF, complement, integrins and angiopoietin are being targeted by new drugs, but VEGF inhibition will likely remain critical to ophthalmic therapy for at least the next decade.

Financial & competing interests disclosure
The author is on the advisory board for Allergan, has acted as a consultant for Boehringer-Ingelheim and is on the advisory board for Regeneron and provided research support (employing institution) for Regeneron. The author had no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues
- Each of the currently used anti-VEGF drugs possesses a high binding affinity for VEGF₁₆₅.
- Anti-VEGF drugs have similar intravitreal half-lives in human eyes (approximately 7 days for ranibizumab and pegaptanib (estimated to be 10 days for bevacizumab) but quite disparate serum half-lives (6 h for ranibizumab to 21 days for bevacizumab).
- The abilities of anti-VEGF drugs to inhibit VEGF-mediated activation of vascular endothelial cells vary between studies but generally adhere to the following hierarchy: aflibercept > ranibizumab > bevacizumab > pegaptanib.
- Intravitreal injections of bevacizumab and aflibercept (but not ranibizumab) decrease serum VEGF concentrations, though the significance of this remains unknown.
- In vitro testing on retina, retinal pigment epithelium and choroidal vascular endothelial cells and in vivo testing in rats, rabbits and monkeys reveal acceptable safety profiles for each drug.
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