Dual acting therapeutic proteins for intraocular use

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Teaser (25-30 words):

Intravitreally injected antibody-based medicines have revolutionised the treatment of retinal disease. Bispecific and dual functional antibodies and therapeutic proteins have the potential to increase the efficacy of intraocular medicines.

Abstract

Antibody-based medicines that target vascular endothelial growth factor (VEGF) are administered by intravitreal injection to treat chronic neovascular retinal diseases. Much ongoing effort is focused on enhancing therapeutic outcome of these medicines. One strategy is the use of dual acting drugs (e.g. bispecific antibodies) to simultaneously bind to more than one intraocular biological target. A dual acting molecule targeting components within the vitreal cavity could also potentially extend vitreous residence time. In this review, the applications of bispecific antibodies within the eye are described with consideration to potential targets, applications and suitable bispecific formats.

Introduction

Intravitreally administered antibody-based medicines targeting vascular endothelial growth factor (VEGF) which causes angiogenesis and neovascularisation have revolutionised the treatment of neovascular retinal diseases in the 21st century [1,2]. Uncontrolled vascularisation and photoreceptor degeneration characterises several posterior blinding conditions including wet age-related macular degeneration (wet-AMD), diabetic retinopathy (DR) and diabetic macular edema (DME) [3]. VEGF is not the only potential target for neovascularisation that may be used to treat chronic intraocular blinding disease [4]. Inflammation is also involved in causing blinding disease (e.g. posterior uveitis) [5]. Dual targeting protein-based therapeutics such as bispecific antibodies (bsAbs) that are capable of interacting with two target epitopes simultaneously [6,7] have the potential to increase the efficacy of intraocular medicines.

The concept of bsAbs has long been known [8] and is envisaged to exploit spatial temporal relationships that are not possible by using a combination or mixture of antibodies [9]. To date, clinical realisation has been achieved in oncology. Blinatumomab was approved in 2015 to treat acute lymphoblastic leukaemia and emicizumab was approved in 2018 to treat haemophilia A [10]. Catumaxomab was approved, but has now been withdrawn for commercial reasons. Blinatumomab is a bispecific T-cell engager (BiTe) molecule comprised of two antibody single chain variable fragments (scFvs) in a molecule with an overall molecular weight of approximately 55 kDa. One fragment of blinatumomab binds to CD19 on a malignant B-cell and the other fragment binds to CD3 on a T-cell to redirect and elicit a cytotoxic

response [11,12]. Emicizumab is a full IgG antibody that binds to blood factors IXa and X to allow the coagulation cascade to continue in the absence of sufficient amounts of factor VIII [13].

Drug combination versus dual acting molecule

Drug combination strategies are widely used in medicine, e.g. oncology, and infection [14]. Intravitreal injections are invasive and carry some risk, so intraocular combination strategies would probably need to be formulated as fixed dose combinations to minimise the number of intravitreal injections. Disadvantages of fixed dose combinations include a lack of dosing flexibility and difficulties to identify adverse reactions. The volume of an intravitreal injection is 50 μL, which is a very small volume for a combination of protein-based drugs at sufficient individual doses while minimising risks of protein misfolding and aggregation. In spite of these limitations, as described below, combinations have been evaluated, but have yet not progressed to clinical registration [14–18].

The key challenge in developing intraocular medicines is to ensure there is no ocular toxicity [19–22]. The eye is susceptible to inflammation which can be caused by immunogenicity to the therapeutic protein and possible protein aggregation. The production of anti-drug antibodies (ADAs) and inflammatory responses [19,23] are damaging and sight-threatening and must be avoided since intraocular tissues are delicate and non-regenerative. It is known that the eye can be susceptible to endophthalmitis following injections [24] Proteins that are modified, for example by PEGylation, must be manufactured to the highest standard as evidenced by the recent regulatory failure and withdrawals of Abicipar pegol [25]. The anti-VEGF PEGylated aptamer, pegaptanib sodium appeared to be well tolerated [26] although its use decreased after the clinical introduction of ranibizumab and aflibercept. Ocular tolerability and safety profiles should be thoroughly assessed. Preclinical and clinical studies must carefully designed [21] to minimise and to quickly observe any adverse reactions related to the protein of interest [22]. Long term studies moving from branded to biosimilar protein therapeutic must also be conducted [27]. In terms of bsAbs for development in retinal therapy, there are ongoing studies in preclinical stages with only one bsAb i.e. faricimab targeting VEGF and angiopoietin-2 (ANG-2) in full clinical development to treat diabetic macular edema (DME) [28,29] and wet-AMD [30–32].

Considering current anti-VEGF biologics require long-term monthly or bimonthly injection, there is also a need to reduce the frequency of intravitreal injections to treat chronic intraocular conditions. There has been near exponential growth of intravitreal injections since 2007 [33] but the need for repeated intravitreal administration is difficult for patients where compliance decreases after the first year of treatment [34–36]. Minimising the cumulative number of intravitreal injections is also important due to the potential of harmful effects to ocular tissues [37]. Chronic ocular hypertension has been associated with repeated intravitreal anti-VEGF injections [38]. Dual acting biologics could potentially be developed where one function is to display increased affinity to tissue in the posterior cavity to slow clearance from the vitreous (affinity targeting) and the other function of the molecule would be to bind to a therapeutic target.

The scope of this review is first to describe intraocular targets that could be considered for the development of dual acting biologics and how bispecific molecules might be also used to increase duration of action. We also briefly describe different bispecific formats.

Current progress of key intraocular targets
Targets to inhibit neovascularisation

VEGF is a proven clinical target for a number of different indications. Since its first discovery as an angiogenic factor in late 1980s [39], several medicines have been developed as VEGF inhibitors in oncology. To date three antibody-based therapies targeting VEGF for intraocular use have been approved, i.e. ranibizumab (Lucentis®), aflibercept (Eylea®) and brolucizumab (Beovu®), and 1 non-antibody-based therapy, i.e. pegaptanib (Macugen®). Bevacizumab (Avastin®) is also widely used off-label to treat intraocular neovascularization. These medicines target epitopes to different VEGF subtypes which have been described in several accessible reviews [40–43]. .

Pegaptanib (Macugen® 2004), is a PEGylated ribonucleic acid (RNA) aptamer that binds with high affinity to VEGF-A (VEGF$_{165}$) to its heparin-binding site [44]. Binding to the heparin-binding site of VEGF$_{165}$ does not fully prevent the binding of VEGF to VEGFR-2, resulting in poor clinical efficacy compared with the other anti-VEGF agents. Unlike pegaptanib, ranibizumab (Lucentis®, 2006) is an antibody antigen-binding fragment (Fab) that binds to VEGF-A to its receptor binding region and inhibits VEGF binding to its receptor, VEGFR2 [45]. Aflibercept (Eylea®, 2011) is

a fragment crystallisable (Fc)-fusion protein that comprises the Fc region of an IgG_1 fused to two copies of the extracellular domain-2 of VEFGR-1 linked to domain three of VEGFR-2 [46]. Aflibercept, also called VEGF-trap, has shown a wider binding capacity (VEGF-A, VEGF-B and placental growth factor (PIGF)) and higher VEGF binding affinity compared to ranibizumab [46]. The patents on ranibizumab and aflibercept will expire in the US in 2020 and in Europe in 2022 and 2025 respectively [47]. A ranibizumab biosimilar called razumab has been clinically used in India since 2015. Other biosimilars (FYB 201, Xlucane, PF582, CHS3351, SB11, BCD300) are in different stages of clinical trials [47]. Aflibercept biosimilars (ABP 938, ALT-L9, M710, CHS-2020) are currently in different phases in clinical trials by different pharmaceutical companies in US and South Korean (e.g Amgen, Alteogen, Momenta, Coherus Bioscience).

Bevacizumab (Avastin®, 2004) is a full IgG_1 that binds to VEGF-A. It is formulated and approved for treatment of colorectal cancer and other oncology related diseases, but is used off-lable usually after pharmacy fractionation into syringes to treat wet-AMD [48]. No difference in visual acuity was observed compared to ranibizumab during multi-center randomised controlled clinical trials [49–51]. There are biosimilars to bevacizumab that are either approved or in clinical trials, but their use is more suitable in oncology rather than ophthalmology.

Brolucizumab (Beovu®, 2019) is a humanised scFv (molecular weight of ~26 kDa) capable of binding to three isomers of VEGF-A ($VEGF_{110}$, $VEGF_{121}$ and $VEGF_{165}$) to prevent their interaction with both VEGFR-1 and VEGFR-2 [52]. An intravitreal injection comprises 6 mg of brolucizumab in a single 50 μL dose is approximately 10 times greater on a molar basis than aflibercept and 20 times greater than ranibizumab. The increased molar dose of brolucizumab is thought to allow administration once every three months after completion of a dose loading period comprised of three monthly injections [52]. Brolucizumab has only recently been approved [53], although post marketing concerns over safety have been reported to the American Society of Retinal Specialists (ASRS) and case studies [54,55] have subsequently been published [56].

Abicipar pegol is a designed ankyrin repeat protein (DARPin) targeting VEGF-A which is conjugated to poly(ethylene glycol) (PEG, 20 kDa). DARPins are adapted from naturally occurring ankyrin repeat units, and are α-helical scaffold proteins with

small molecular weights [57]. A DARPin with seven binding units has a molar mass of only 26 kDa, which is less than a Fab such as ranibizumab (~50 kDa). Abicipar has an exceptionally high picomolar potency and better stability compared to the approved anti-VEGF antibodies in angiogenesis models of the eye [58]. Intraocular inflammation was reported during the phase II and III trials [59,60] were thought to be from manufacturing impurities [61]. While the FDA has accepted a Biologics License Application (BLA) of abicipar [62] the agency did not approve its clinical use (date June 2020) [63].

Other anti-VEGF biologics currently in late stage clinical development include conbercept, OPT-302 and KSI-301. These biologics are capable of binding to VEGF isomers to inhibit binding of VEGF to VEGF-receptors resulting in neutralisation of VEGF signalling pathways (e.g angiogenesis and neovascularisation). Conbercept and OPT-302 are Fc fusion proteins analogous in their structures to aflibercept. Conbercept which has been marketed in China since 2014 and is currently in phase III studies in the US, comprises of 2 copies of domain 2 of VEGFR-1 linked to domains 3 and 4 of VEGFR-2. The Fc region in OPT-302 is fused to two copies of extracellular domains 1-3 of VEFGR-3. OPT-302 inhibits VEGF-C and -D and is currently in Phase IIb trials for the treatment of neovascular AMD in combination with anti-VEGF-A molecules [64,65]. Complete blockade of the VEGF signalling pathway may be achieved through inhibition of VEGF-A along with VEGF-C and -D signalling pathways. This is suggested to have better results in neovascular regression compared to inhibition of single VEGF-A pathway [66].

KSI-301 is an anti-VEGF IgG1 antibody that is covalently conjugated to a high molecular weight phosphorycholine biopolymer recently entered phase II clinical trials for the treatment of wet-AMD. KSI-301 is designed to block all VEGF-A isomers [67] and comprises of IgG1 antibody that covalently conjugated to a high molecular weight. The concept is to increase intraocular duration of action by leveraging hydrodynamic size and molar dose [68]. KSI-301 appeared to be 3.5 fold greater equivalent molar dose than aflibercept [68].

To augment therapies to inhibit neovascularisation [69], other possible clinical targets have emerged (Table 1) including neutralising platelet-derived growth factor-B (PDGF-B), PDGF receptor-B (PDGFR-B) [70] and angiopoietin receptors (Tie-2) [71–73] are also being explored to treat ocular neovascularisation [4]. Targeting vascular pathways such as tyrosine kinase receptor 2 or angiopoietin receptors (Tie-2) and

platelet-derived growth factors (PDGF and TGF-B) have shown promising results in regression of neovascularisation and vessel stabilisation. The Tie-2 receptor, like the VEGF receptor, is expressed in the endothelium and plays an important role in vascular network progression. Angiopoietin-2 (ANG-2) is a ligand that binds to the Tie-2 receptor and acts as a pro-angiogenic factor promoting angiogenesis in conjugation with VEGF. ANG-2 has also been shown to enhance retinal blood vessel sensitivity to the angiogenic effects of VEGF [74]. Nesvacumab is a monoclonal antibody against ANG-2 for the treatment of DME.

PDGF is another growth factor that stimulates blood vessel formation, proliferation and angiogenesis, and may contribute to neovascularisation in wet-AMD [75]. PDGF binds to PDGFR-A and PDGFR-B which are tyrosine kinase receptors that are expressed in vascular smooth muscle cells and pericytes. Pegleranib (Fovista, Ophthotech) is a PEGylated aptamer that binds to PDGF-BB to prevent its binding to PDGFR-B [76]. Inhibition of PDGF binding to PDGFR-B causes pericytes to be stripped from vessels that have been abnormally formed, leading to their regression [77]. Another example is the development of rinucumab, an IgG4 monoclonal antibody, that targets PDGF-R. These findings suggest that developing biologics inhibiting the PDGF or PDGFR pathway might be valid targets for the treatment of ocular neovascularisation.

Drug combinations to target multiple ligands or receptors is successfully widely employed in different areas of medicine, e.g oncology and infection. In the case of ocular neovascularisation, great efforts have been made to design and formulate drug combinations with multiple targets with several examples in phase II trials, but so far, these have not been translated into successful phase III trials. For example, targeting PDGF and VEGF has been examined with rinucumab (anti-PDGF IgG4 co-formulated with aflibercept) and E10030/pegpleranib (Fovista in combination with ranibizumab) in phase II and III trials for treatment of wet-AMD respectively [18] but failed to show a benefit over anti-VEGF monotherapies.

Inhibition of Ang-2 in combination with VEGF has also been suggested as a potential combination for treating neovascularisation [78]. Two phase II trials have been conducted using nesvacumab (anti-ANG-2 antibody) and aflibercept for the treatment of wet AMD (ONYX) and DME (RUBY). Results of these trials showed no statistical difference between best corrected visual acuity and central subfield thickness compared to aflibercept monotherapy [15,16].

While the vitreous is an acellular compartment of the eye, there are cellular targets that are present in the retinal tissue. Tissue factor (TF) is a surface receptor target for coagulation factor VII which initiates the extrinsic coagulation pathway, plays an important role in retinal neovascularisation [79]. In a normal healthy eye, TF is not expressed by cells but is expressed in response to inflammation by vascular endothelial cells, monocytes and macrophages [80]. It has been shown that intravitreal injection of anti-TF monoclonal antibody results in reduction of CNV in a mouse model. Based on this finding, inhibition of TF was reported as a potential therapeutic target to treat retinal neovascularisation, with the ICON-1 molecule having completed phase II trials for treatment of choroidal neovascularisation (CNV) [81–83]. ICON-1 is an Fc-fusion protein comprising of two human factor VII domains, conjugated to a human Fc fragment which selectively binds to TF destroying pathological vessels [84].

Integrin is another emerging intraocular target which plays an important role in regulating cellular adhesion, kinase signalling pathways, endothelial cell migration, apoptosis and VEGFR-2 activation leading to network formation during vascular development [85]. Inhibition of integrin is of interest because of its potential to have a therapeutic role in inhibiting CNV in AMD patients. In general, integrins are transmembrane proteins that bind to extracellular matrix (ECM) proteins such as laminin, fibronectin and collagen. Integrin a5b1 is a fibronectin receptor involved in endothelial cell migration and proliferation [86]. Volociximab is a monoclonal antibody that binds to fibronectin to inhibit its binding to integrin a5b1. A phase I trial assessing the safety profile of volociximab was completed in 2012 with positive results [87], however, to date no further studies have been undertaken to investigate volociximab for the treatment of AMD.

The bioactive lipid sphingosine-1-phosphate (S1P) was thought to be another potential intraocular target [88] for which an anti-S1P monoclonal antibody (iSONEP or Sphingomab) was developed by Lpath Inc. S1P is a circulating lipid mediator generated from metabolism of cell membranes and is involved in multiple mechanisms of action in inflammation and angiogenesis [89]. However, iSONEP failed to progress past phase II trials because it did not show any significant improvement in visual acuity of patients with wet AMD.

Targets to inhibit inflammation

Intraocular inflammation contributes to many disease pathologies including neovascularisation and uveitis. Steroids are used to treat uveitis but their efficacy is limited. Biologics to target a specific cell type or pathway are being explored for the treatment of autoimmune uveitis. Studies in photoreceptor apoptosis have shown that proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (IL-6, IL-6R, IL1-β, IL-17A and IL-23) [90] could play an important role in the progression of neovascular and inflammatory diseases. While targets to treat inflammation have begun to emerge, most investigations have been conducted by administering the antibodies parenterally rather than by intraocular injection since intravitreal formulations have not yet been developed. Adalimumab is a fully human monoclonal antibody against TNF-α that has been approved by the FDA and European Medicines Agency (EMA) to treat non-infectious, posterior and pan-uveitis in adults and children over 2 years old [5,91].

Tocilizumab targets the interleukin 6R (IL-6R) and is approved for the treatment of rheumatoid arthritis, and is currently in phase II trials for the treatment of refractory Behcet's uveitis. Elevated concentrations of IL-6 has been detected in the vitreous of patients with posterior uveitis [92,93]. Safety and efficacy of another anti-IL-6R antibody called sarilumab is being evaluated in phase II trials for posterior segment non-infectious uveitis. Satralizumab, an anti-IL6R antibody, has recently been developed for other inflammatory related disease, neuromyelitis optica spectrum disorder as one of a rare neurological brain condition caused by inflammation in optic nerve.

Canakinumab and gevokizumab are two antibodies targeting IL-1β. Canakinumab is approved for treatment of two forms of cryopyrin-associated periodic syndrome (CAPS), as an intravenous formulation has recently completed (in July 2020) the phase II trails for Behcet's associated uveitis. Gevokizumab is being developed by the XOMA Corporation, but unfortunately has failed to meet its primary endpoint in phase III trials [94] for the treatment of uveitis.

Other proinflammatory targets such as IL-17 and IL-23 have been shown to contribute to the progression of uveitis disease [95] leading to the development of anti-IL-17 (secukimumab) and anti-IL-23 (ustekinumab) antibodies. While secukimumab failed to progress phase III [96] ustekinumab is currently in phase II trials [97] (Table 1). Insulin like growth factor (IGF-1R) has also been examined and an anti-IGF-R1

antibody, teprotumumab, has been approved to treat thyroid eye disease as muscles and fatty tissues behind the eye become inflamed [98].

The antibodies targeting anti-inflammatory cytokines are given in high does (e.g 5 mg/kg) because they are systemically administrated by parenteral route. High doses are necessary to achieve some biodistribution within the eye. Intravitreal dosage forms have not been developed, so safety concerns remain including an increased incidence of endophthalmitis. Development of intravitreal dosage forms would better ensure that a reproducible dose could be delivered intraocularly. Since intravitreal doses are low relative parenteral doses, there would be less systemically associated side effects.

Dual therapeutic targeting

Faricimab (represented in **Figure 1A**) is an IgG antibody with the capability to bind two therapeutic targets. Faricimab which comprises one Fab with specificity to VEGF and another Fab with specificity to ANG-2, is in phase III trials for the treatment of wet-AMD [30] and DME [29]. Blocking two soluble targets, dual acting antibodies could combine the activities of two pathway-modulating molecules into one for enhanced efficacy. Faricimab was developed as researchers began to look beyond anti-VEGF monotherapies due to poor response and recurrence of disease [99]. Benest et al. [100] found that a reduction in ANG-2 concentration strongly reduced the effect of vascular leakage upon administration of VEGF as ANG-2 upregulates the neovascularisation effects of VEGF. Faricimab was optimised for use in the eye by abolishing its Fc binding interactions with FcγR and FcRn. This was achieved by exchanging the amino acids required for Fc related interactions. Phase III Trials (YOSEMITE, TENAYA) are underway to compare efficacy and pharmacokinetics of faricimab using a 8 weeks dosing interval with aflibercept for both wet-AMD and DME treatment [29,78]. Considering the need to avoid ocular toxicity and develop more efficacious drugs, as a bispecific faricimab appears to demonstrate a favourable safety profile. There were no reports of intraocular inflammation during the phase II BOULEVARD trial up to 36 weeks [28]. However, data obtained during the phase III clinical trials will provide more detail about the safety profile of faricimab.

Another example for dual acting antibody with two therapeutic targets is the development of a molecule called valpha which was investigated by Korea Advanced Institute of Science. Valpha is a Fc-based bispecific molecule that targets VEGF and TNF-α [101] It comprises of soluble VEGF and TNF-α receptors, which are fused to a

Fc IgG region. A study showed that when compared to two control monospecific (anti-VEGF aflibercept and anti-TNFα etanercept) therapies, valpha has the potential to increase treatment effectiveness due to its dual targeting approach, and a favourable pharmacokinetic profile [101]. Valpha has the potential to be a cost-effective strategy for the treatment of AMD. However, it appears that no further development has been conducted on this molecule since 2011. While the reasons for the lack of development have not been publicly disclosed and there is no indication of a lack of efficacy, the presence of the Fc region in the bispecific format could lead to ocular or/and systemic cytotoxicity, which will be reviewed in following section. The lack of Fc function is important because upon clearance from the eye, there will be no Fc mediated recycling or effector function which may reduce systemic safety risks.

Dual action molecules designed for increased duration of action

There is a recognised need to increase the duration of action of intravitreally administered medicines [36,102–104]. There is often reduced compliance by patients after the first year of treatment [34,69], especially patients that have not previously participated in a clinical trial [35,36]. Strategies to develop complex formulations of therapeutic proteins [105–107] have been considered, but these strategies must address the challenges to maintain protein stability [108–110] and ocular tolerability [19,23]. The Port Delivery System (PDS) is a refillable reservoir for the long-term administration of ranibizumab that is currently undergoing Phase III trials [111,112]. The PDS is implanted in the sclera with an extra scleral flange with a self-sealing septum designed to allow access to the reservoir to remove and replenish drug in a clinical setting using aseptic technique. While this strategy avoids the need of an intravitreal injection, the implantation of the PDS must be accounted for when considering the range of possible adverse reactions [113].

Another strategy to potentially increase the residence time of a therapeutic protein in the vitreous is for the protein to associate or bind to a tissue component within the vitreous cavity. As a large molecular weight molecule possessing charge, a therapeutic protein generally clears via aqueous outflow after diffusing from the vitreous into the anterior chamber where convective flow clears into the conjunctiva [114,115]. If there is an absence of interactions with ocular tissue in the posterior cavity, the clearance of biotherapeutics is primarily dominated by molecular size because molecules diffuse from the viscous vitreous gel [106,114–116]. Charge and

hydrophobicity characteristics of therapeutic proteins appear to make little contribution to the elimination time from the vitreous compared to the influence of the size of the therapeutic protein in solution (i.e. hydrodynamic radius) [116]. The vitreous often becomes less viscous as we age, so the diffusion times, and thus clearance times can show considerable interpatient variation [104,117–119].

Affinity drug delivery strategies have attracted interest [120,121] and transient interactions between a therapeutic protein and an endogeneous intraocular target [122] can in principal be used to reduce clearance times from the vitreous cavity. After a loading dose has been administered, a therapeutically beneficial maintenance dose at low concentration can in principle be achieved by an affinity strategy.

Extending vitreous residence time by affinity can either involve binding to an endogenous target in the posterior cavity (e.g. hyaluronic acid (HA), collagen) (Table 2) or possibly to an exogenously administered target (e.g. binding to hydrogel or implant) [123]. Some relevant tissue component binding constants have been reported [124] and the amounts of possible vitreous targets have also been described [122].

Binding to a target in the vitreous must not cause any ocular toxicity or interference with vision. Also, to ensure rapid systemic clearance after the drug exits the eye, the selected anchoring target in the vitreous should ideally not be present in the blood compartment. For example, small amounts of albumin have been found in the healthy vitreous and the amount of albumin may be higher in some disease conditions such as diabetic retinopathy [125]. The challenge is that albumin in the blood compartment would then act to extend the circulation time of the drug after clearing from the eye. While targeting albumin in circulation is well known and is a clinically proven strategy [126–128], utilising albumin in the eye may be limited if the drug is also not required systemically after clearing from the eye.

Researchers from Novartis described a bispecific molecule comprised of an anti-VEGF Fab fused with the HA-binding component derived from hyaladherin [122]. Results showed that HA binding anti-VEGF adducts displayed ~3-4 fold longer half lives in rabbit and monkey eyes than non-HA binding controls. Inhibition of VEGF-induced vascular leak was also 3-4 times longer in animal models with the HA binding bispecifics.

Another example has been described by Roche [129] that reports the preparation of a recombinant fusion protein (peptide linker) with the first binding site (Fab or scFv) has therapeutic action targeting VEGF, and the second binding site

specifically binds to type II collagen (scFv) [129]. The bispecific molecule increased diffusion time by 2.7 times in phosphate buffered saline containing collagen and 3.2 times in vitreous fluid compared to the therapeutic Fab without the affinity binding moiety.

Targeting heparan sulphate proteoglycans (HSPGs) has also been suggested as another affinity target to prepare a dual acting molecule [130]. HSPGs are cell surface glycoproteins of heparan sulphate found on retinal pigment epithelial (RPE) cell surface and also in the ECM and basement membrane. Heparin binding domains (HBDs) are the ligands binding to HSPGs to regulate cell activities. Fusion of HBDs to the aflibercept in "sticky-trap" molecule, resulted in prolonged drug retention within the vitreous for 12 days longer than aflibercept [131].

The concept of affinity targeting in the eye has also been extensively described with melanin, but mostly for low molecular weight molecules. Melanin is the most common light-absorbing pigment and is located in melanosome vesicles within the retinal pigment epithelium (RPE) cells [132] and approximately 6-8 mg of melanin is present in the ocular tissues [133]. Urtti and coworkers have reported extensively on drug-melanin binding [104,134–139]; and have recently established a correlation between *in vitro* binding and *in vivo* pharmacokinetics [140]. Drugs can bind to melanin altering its pharmacologic and pharmacokinetic profiles [141] by forming a reservoir to prolong residence time [142,143]. Intravitreal administered low molecular weight molecules that bind to melanin have been reported [138,144,145] namely β-blockers, celecoxib and chloroquine. Melanin binding is more pronounced with lipophilic drugs (e.g. beta-blockers) [146–148].

The duration of action of a drug may also be extended by developing an exogenously administered binding target (or anchor). Shoichet and coworkers have explored the affinity between a protein and a hydrogel [121,123,149–152]. In one study by Delpace et al[123], ciliary neurotrophic factor (CNTF) was expressed as a model protein with neuroprotective effect on the retina and then fused with Src homology 3 (SH3) domain. The CNTF-SH3 molecule was then formulated with a hydrogel system (hyaluronic acid and methylcellulose). The hydrogel composition was modified with an SH3 binding peptide allowing reversible binding of the fusion protein (CNTF-SH3) to the gel matrix [123]. Intravitreal injection to the retina, the *In vivo* activity was similar

to that of commercial CNTF; however, there was a lack of prolonged effect for CNTF-SH3, due to insufficient protein being present at 7 days[123].

Bispecific molecular motifs include IgG and non-IgG formats

The IgG format (e.g faricimab Figure 1A) is not the only molecular format that is being examined for use as a bispecific, dual functional therapeutic protein for intraocular use. Fc-fusion (e.g. aflibercept) and Fab (e.g. ranibizumab) possess elements of the IgG format. Non-IgG formats that have already been referred to are brolucizumab, which is a scFv and abicipar which is a DARPin. These and other non-IgG formats [153–155] along with other molecules described in the patent literature [156,157] may also have potential intraocular applications. Other non-IgG formats (such as nanobodies, Diabodies, BiTEs and DARTs) that have been developed for use in oncology, may not be ideal for systemic use due to suboptimal clearance rates from the blood compartment. These may potentially emerge for intraocular use.

As with antibody-based medicines that are considered to have be related to the IgG motif (e.g. IgG, Fab, Fc-fusion), the non-IgG formats are protein-based molecules that are large sized (> 10 kDa), charged molecules in solution. Although the Fc function in an IgG molecule can be disabled by molecular engineering (e.g. faricimab), the non-IgG formats do not have an Fc region.

A bispecific DARPin targeting VEGF and PDGF is currently in pre-clinical development for ocular diseases [158] and has not yet entered clinical trials. In this molecule, two different DARPins are linked via peptide linkage. The structure of a bispecific DARPin is shown in **Figure 1B**. Nanobodies are derived from camelids including camels, llamas and alpacas [159]. They are comprised of heavy chain variable regions and have molecular weights as low as 13 kDa. Nanobodies share a number of advantages with DARPins that may be important for intraocular used including high solubility, stability and small molecular size [159]. Bispecific nanobodies can be synthesised linking two different nanobodies with a shorter linker sequence (**Figure 1C**). Despite these advantages, there are no bispecific nanobodies yet being developed for ocular diseases. However, an interesting bispecific nanobody, called BI 836880, that blocks VEGF and ANG-2 has been described for oncology and is currently in phase I clinical trials [160].

Diabodies, BiTEs and DARTs are other non-IgG formats that can be made into bispecific molecules. These formats comprise scFvs linked together by different

arrangements. BiTE (bispecific T-cell engager) are non-endogenous molecules comprised of two scFvs and have a molecular weight of approximately 50kDa. They are manufactured with peptide linking two scFvs derived from different monospecific monoclonal antibodies [161], in contrast to diabodies in which the variable fragments contain light and heavy chains from the same antibody. A key to the functionality of BiTE molecules is a freely rotatable peptide linkage. The freely rotatable linkage enables the scFvs to interact with targets on different cell surfaces or while in solution. The DART platform comprises two scFvs which contain interchain linkers and covalent bonds. This non-endogenous configuration limits the rotation of the antigen binding domains in contrast to the free rotatable BiTE [162]. All three formats (diabodies, BiTE and DART) have entered clinical development in oncology with the most successful (to date) being blinatumomab (Blincyto). Currently these drugs are formulated as parenteral dosage forms and are not yet made into high concentration formulation [163] required for ocular application. In addition, there are general concerns regarding the stability, toxicity, and immunogenicity caused by peptide linker for diabodies and BiTE molecules which may pose challenges for intraocular use.

Other bispecific formats that have shown promising results for ocular indications are bispecific aptamers and bispecific $F(ab)_2$. Bispecific aptamers such as SOMAmers which target VEGF and PDGF have been made by SomaLogic, Inc [164,165]. Bispecific $F(ab)_2$ is another design with the potential benefit of being a "human-like" bispecific mimicking a human IgG structure without the Fc **(Figure 1D)**. The $F(ab)_2$, could be an interesting format to peruse because of the success of ranibizumab for treatment of wet-AMD. The inclusion of two Fabs and the lack of the Fc in the $F(ab)_2$ bispecific, could enhance the targeting properties of these molecules while maintaining the favourable rapid systemic pharmacokinetic profile of the Fabs [166].

In a bispecific $F(ab)_2$, two different Fabs can be linked together either by a peptide linker or polymer linker. Two Fabs could have dual therapeutic function or affinity-based function with one Fab binding to a vitreous specific tissue and the other Fab having a therapeutic function. We have recently synthesised a monospecific $F(ab)_2$ like format, a Fab-PEG-Fab molecule (FpF) using a recombinant-chemical approach. The FpF molecule shows many similarities to an IgG molecule (e.g. solution size and binding affinity). An FpF is synthesised by site-specific conjugation of two Fabs using a safe poly (ethylene glycol) (PEG) linking molecule **(Figure 2A)** [167–

170]. The Fab interchain disulfides in the FpF mimetics, are stabilised by reannealing disulfide bridging conjugation. The presence of PEG reduces the propensity of the FpFs to aggregate. The FpFs displayed slower dissociation rate constant (k_{off}) compared to the parent IgG, the binding affinity (K_D) for FpF appeared to be similar as IgG for both VEGF and TNFα [167–170]. The anti-TNFα FpF displayed comparable anti-inflammatory activity as infliximab in an uveitis mouse model [170]. Exploiting reduced dissociation rates (k_{off}) of therapeutics may be a viable approach to increase efficacy within ocular tissue. We have also prepared Fc-fusion mimetics called RpRs (receptor-PEG-receptor) with similar binding properties. We are currently developing the bispecific FpFs (Figure 2B) designed for intraocular applications [171].

Future prospects: As with any therapeutic, target selection is a first critical step for the development of dual acting, or bispecific biotherapeutics. Target selection is compounded by the need to select two different targets that together will bring clinical benefit. Bispecific therapeutics are clinically proven in oncology where they can exploit spatial temporal relationships that are not possible using a combination of constituent drugs. The use of intravitreally administered drug combinations has been limited to date by not meeting efficacy endpoints and may be further limited by formulation challenges and regulatory requirements. Challenges with the scale of the manufacturing process, characterisation and product stability slow their clinical development. Therefore there may be more opportunities than anticipated based purely on spatial temporal relationships to develop bispecific biotherapeutics for intraocular use. Future development of bispecifics for intraocular applications requires that there is no ocular toxicity caused by the therapeutic. All molecular formats will require thorough evaluation for intraocular use.

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Figure Legends:

Figure 1 Structures of five bispecific antibodies (IgG and non-IgG formats) for ocular indications:
(A): A bispecific antibody produced using CrossMAb technology. **(B):** A bispecific DARPin containing two DARPins linked by a peptide linker (adapted from PDB entry 5O0U). **(C):** A bispecific nanobody molecule, two heavy chain only antibody fragments linked together by a peptide linkage. **(D):** A bispecific F(ab)2 molecule, two Fab regions are linked via a hinge disulphide bond. **(E):** A bispecific aptamer molecule containing two oligonucleotide aptamers linked together via a peptide linkage (adapted from PDB entry 2AU4).

Figure 2 Structures of monospecific and bispecific FpF molecules:
(A) A monospecific FpF in which two identical Fabs are covalently bound to end of a protein dimerisation reagent to form a homodimer. **(B)** A bispecific FpF in which two different Fabs are covalently bound to end of a protein dimerisation reagent to form a heterodimer.

Figure 1

A

Bispecific CrossMAb e.g. Faricimab

B

Bispecific DARPin

C

Bispecific nanobody

D

Bispecific F(ab)2

E

Bispecific aptamer
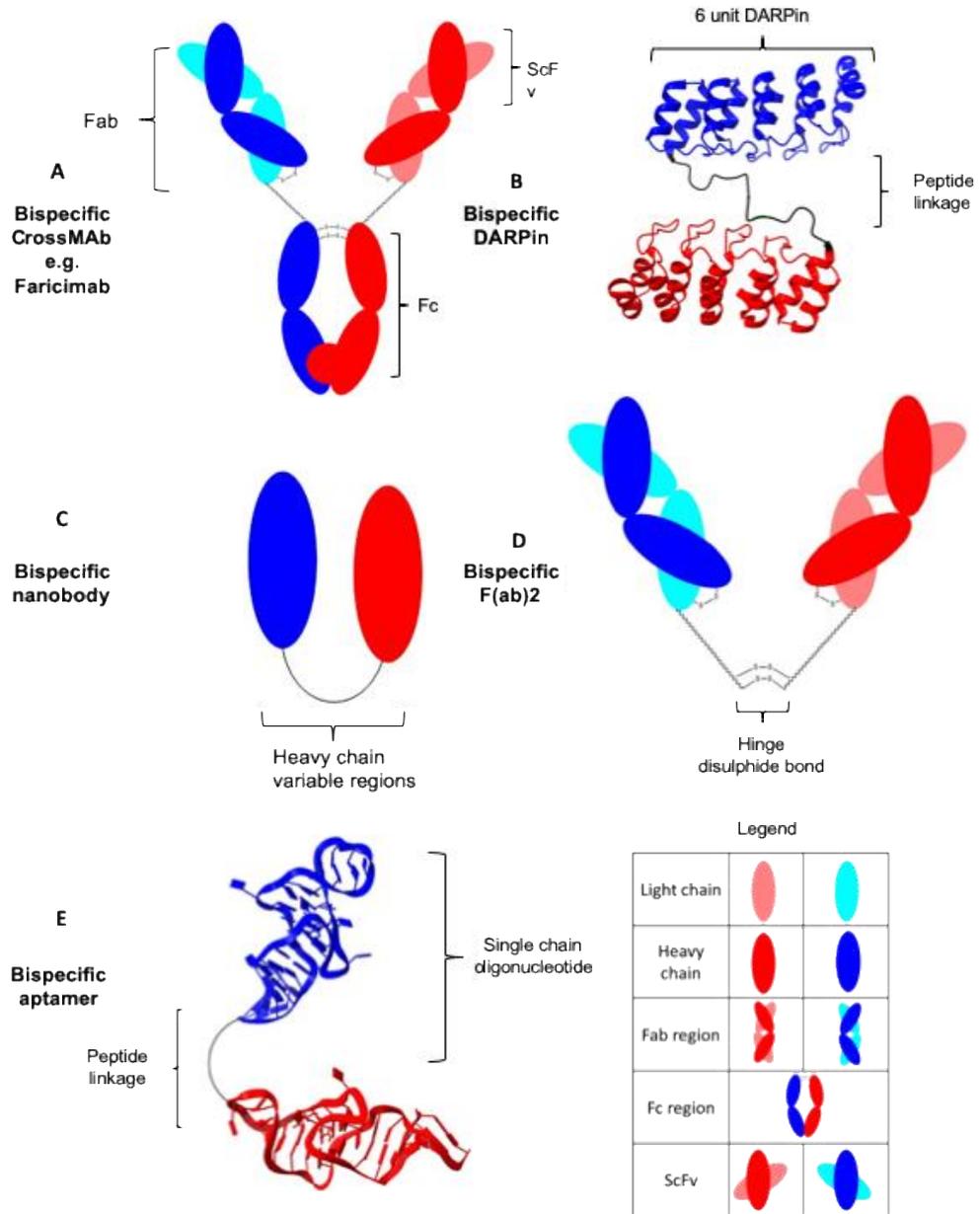
Figure 2
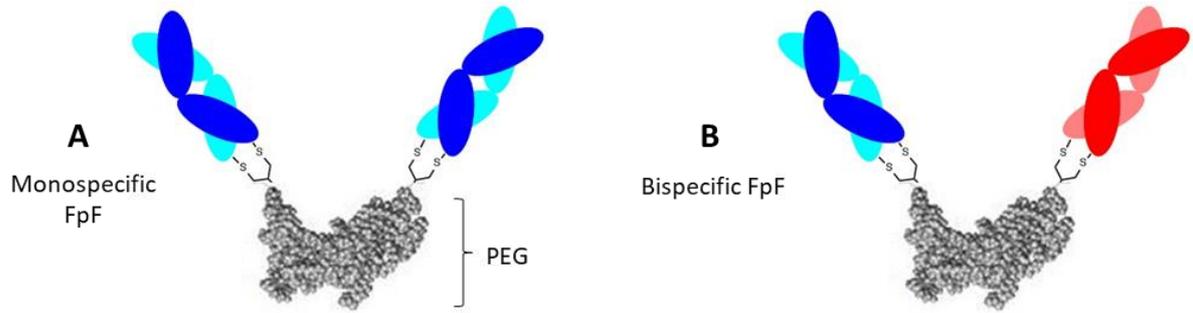
Table 1. Druggable targets in clinical development for treatment of wet-AMD and non-infectious uveitis.

Type of therapeutic target	Therapeutic target	Name of drug	Format	Clinical progress	Clinical dose (mg)	Molar dose per injection	Company
Neovascular ligand	VEGF	Abicipar Pegol (34 kDa)	PEGylated DARPin	Phase III [1,2] (Rejected by FDA, July 2020 [3])	2 mg (50 µL IVI)	3- 4	Molecular Partners / Allergan
		KSI-301 (950 kDa)	IgG1 biopolymer conjugate	Phase II [4]	5 mg (by the weight of antibody) (50 µL IVI)	3- 4	Kodiak
		OPT-302 (115 kDa)	Fc-fusion	Phase IIb [5]	2 mg (50 µL IVI)	1	Opthea
		Conbercept (140 kDa)	Fc-fusion	Phase III [6–13]	2 mg (50 µL IVI)	1	Chengdu Kanghong
	PGDF	Rinucumab (150 kDa)	IgG4	Phase II in combination with anti-VEGF drug (no benefit over VEGF monotherapy) [14–16]	3 mg (50 µL IVI)	1.0	Regeneron
	ANG-2	Nesvacumab (150 kDa)	IgG1	Phase II in combination with anti-VEGF drug (discontinued)[17]	6 mg (50 µL IVI)	2	Regeneron
	VEGF/ANG-2	Faricimab (150 kDa)	Bispecific CrossMab	Phase III [18–21]	6 mg (50 µL IVI)	2	Roche
Neovascular receptor	TF	HI-con1 (115 kDa)	Fc-fusion protein	Phase II [22,23]	0.5 mg (50 µL IVI)	0.3-0.4	Iconic Therapeutics
	Fibronectin receptor	Volociximab (150 kDa)	IgG1	Phase I [24]	2.5 mg (50 µL IVI)	1	Ophthotech
Inflammatory ligand	TNF-a	Adalimumab (150 kDa)	IgG1	Approved for non-infectious Uveitis [25,26]	Up to 40mg (SC)	-	Abbott
	IL-6R	Tocilizumab (150 kDa)	IgG1	Phase II for non-infectious Uveitis [27]	8mg/kg (IV) 162mg (SC)	-	Roche
		Sarilumab	IgG1	Phase II [28]	200mg (SC)	-	Sanofi/Regeneron
	IL-1β	Canakinumab	IgG1	Phase II completed in 2019 [29]	300 mg (IV)	-	Novartis
		Gevokizumab	IgG1	Phase III (failed to meet primary endpoint) [30]	60mg (SC)	-	XOMA/Novartis
	IL-17A	Secukimumab	IgG1	Phase III (failed to meet primary endpoint) [31]	Up to 300mg (SC)	-	Novartis
	IL-23	Ustekinumab	IgG1	Phase II [32,33]	90mg (SC) Up to 520mg (IV)	-	Janssen

Table 2. Affinity bispecific antibodies in preclinical development for the treatment of ocular neovascularisation diseases

Target combination (Therapeutic + Affinity)	Name of drug	Format	Group/Company
VEGF + hyaluronan	NVS24	Fab + HA-binding domain of TSG-6	Novartis [34]
VEGF+ HSPGs	Sticky-trap	Fc-fusion (VEGF trap fused with HBDs)	Michael et al [35]
VEGF+ Collagen II	Undisclosed	Fusion protein (scFv fused with Fab)	Roche [36]

Abbreviation: (HBDs, Heparin-binding domains; HSPGs, Heparan sulphate proteoglycans; TfR, Transferrin receptor).

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