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Dual-acting therapeutic proteins for intraocular use

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

Highlight statements (4 to 5 statements):

- Bispecific antibodies and dual acting therapeutic proteins able to bind two therapeutic targets may be a better strategy than considering drug combinations to treat chronic retinal diseases.
- Several targets to treat neovascularisation and inflammation have been identified.
- A dual acting molecule to address a need to increase the duration of action in the vitreous could improve the treatment of chronic retinal diseases.
- The key challenge in developing intraocular medicines is ensuring there is minimal ocular toxicity. Maintaining protein stability and avoiding protein aggregation and misfolding are necessary.

Glossary (*containing definitions of key terms used during the review***):**

- Dual therapeutic bispecific antibodies (bsAbs) are molecules capable of binding to two different targets simultaneously
- Affinity dual acting molecules are designed to contain therapeutic and vitreous tissue specific binding domains. The purpose of the binding domain specific to vitreous tissue is to delay ocular clearance of the molecule.
- Neovascular ligands are non-membrane bound soluble targets that bind to cell surface receptors resulting in uncontrolled vascularisation. Multiple ligands are responsible for vascularisation making them potential targets for ocular bispecific antibodies.
- Neovascular receptors are membrane bound druggable targets that interact with ligands, contributing to angiogenesis.
- Inflammatory ligands are proinflammatory cytokines that contribute to ocular inflammatory and ocular neovascularisation diseases.
- Bispecific format suitable for intraocular indication is not limited to the traditional IgG format. Other formats with no Fc function (or null Fc) are being developed including bispecific DARPin, scFvs and $F(ab)_2$.

Abstract

Antibody-based medicines that target vascular endothelial growth factor (VEGF) are administered by intravitreal injection (IVI) to treat chronic neovascular retinal diseases. Much ongoing effort is focussed 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 extend vitreous residence time. In this review, we describe the applications of bispecific antibodies within the eye, with consideration of potential targets, applications, and suitable bispecific formats.

Keywords: Bispecific antibody-based medicine, Ocular bispecific, Therapeutic protein, Anti-VEGF antibody-based medicine, Dual acting molecule, Intravitreal injection.

Introduction

Intravitreally administered antibody-based medicines targeting VEGF, which causes angiogenesis and neovascularisation, have revolutionised the treatment of neovascular retinal diseases [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 can 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 are envisaged to exploit spatiotemporal 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 comprising two antibody single-chain variable fragments (scFvs) in a molecule with an overall molecular weight of ~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 molecules

Drug combination strategies are widely used in medicine, such as in oncology, and infection [14]. IVI is invasive and carries some risk; thus, intraocular combination strategies would need to be formulated as fixed-dose combinations to minimise the number of IVIs. Disadvantages of fixed-dose combinations include a lack of dosing flexibility and difficulties in identifying adverse reactions. The volume of an IVI 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. Despite these limitations, and as described later, 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 antidrug antibodies (ADAs) and inflammatory responses [19,23] are damaging and sight-threatening and must be avoided because intraocular tissues are delicate and nonregenerative. The eye can be susceptible to endophthalmitis following injections [24]. Proteins that are modified, for example by poly(ethylene glycol) (PEG)-ylation, must be manufactured to the highest standard, as evidenced by the recent regulatory failure and withdrawal 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 quickly observe any adverse reactions related to the protein of interest [22]. Long-term studies moving from branded to biosimilar protein therapeutics 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 DME [28,29] and wet-AMD [30–32].

Given that current anti-VEGF biologics require long-term monthly or bimonthly injections, there is also a need to reduce the frequency of IVIs to treat chronic intra-ocular conditions. There has been near-exponential growth of IVIs since 2007 [33], but the need for repeated intravitreal administration is difficult for patients, and compliance often decreases after the first year of treatment [34–36]. Minimising the cumulative number of IVIs is also important because of the potential for harmful effects to ocular tissues [37]. Chronic ocular hypertension has been associated with repeated intravitreal anti-VEGF injections [38]. Dual-acting biologics could 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 would be to bind to a therapeutic target.

Here, we 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 several different indications. Since its first discovery as an angiogenic factor during the late 1980s [39], several drugs 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 one non-antibody-based therapy [i.e., pegaptanib (Macugen®)]. Bevacizumab (Avastin®) is also widely used off-label to treat intraocular neovascularisation. These drugs target epitopes to different VEGF subtypes, and are described in several accessible reviews [40–43]. .

Pegaptanib is a PEGylated RNA aptamer that binds with high affinity to VEGF-A ($VEGF_{165}$) via its heparin-binding site [44]. This binding does not fully prevent the binding of VEGF to VEGFR-2, resulting in poor clinical efficacy compared with other anti-VEGF agents. Unlike pegaptanib, ranibizumab is an antibody antigen-binding

fragment (Fab) that binds to VEGF-A via its receptor-binding region and inhibits VEGF binding to its receptor, VEGFR2 [45]. Aflibercept is a fragment crystallisable (Fc)-fusion protein that comprises the Fc region of an immunoglobulin G_1 (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 with ranibizumab [46]. The patents on ranibizumab and aflibercept expire in the USA 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, and BCD300) are in different stages of clinical trials [47]. Aflibercept biosimilars (ABP 938, ALT-L9, M710, and CHS-2020) are in different phases in clinical trials by different pharmaceutical companies in the USA and South Korean (e.g., Amgen, Alteogen, Momenta, and Coherus Bioscience).

Bevacizumab is a full IgG_1 that binds to VEGF-A. It is formulated and approved for the treatment of colorectal cancer and other oncology-related diseases, but is used off-label, usually after pharmacy fractionation into syringes to treat wet-AMD [48]. No difference in visual acuity was observed compared with ranibizumab during multicentre 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 than in ophthalmology.

Brolucizumab is a humanised scFv (molecular weight ~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 IVI comprises 6 mg brolucizumab in a single 50 µl dose, which is ~ten 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 3 months after completion of a dose-loading period comprising monthly injections for 3 months [52]. Brolucizumab was only recently approved [53], although postmarketing 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, and is conjugated to 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 with 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] and was thought to be from manufacturing impurities [61]. Although the US Food and Drug Administration (FDA) accepted a Biologics License Application (BLA) of abicipar [62], it did not approve its clinical use (June 2020) [63].

Other anti-VEGF biologics currently in late-stage clinical development include conbercept, OPT-302, and KSI-301. These are capable of binding to VEGF isomers to inhibit binding of VEGF to VEGF-Rs, 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 USA, comprises two 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 could be achieved through inhibition of VEGF-A along with the VEGF-C and -D signalling pathways. This is suggested to have better results in neovascular regression compared with inhibition of single VEGF-A pathway [66].

KSI-301, an anti-VEGF IgG1 antibody that is covalently conjugated to a high-molecular-weight phosphorylcholine biopolymer, recently entered Phase II clinical trials for the treatment of wet-AMD. KSI-301 is designed to block all VEGF-A isomers [67] to increase the intraocular duration of action by leveraging hydrodynamic size and molar dose [68]. KSI-301 appeared to exert a 3.5-fold greater effect compared with the equivalent molar dose of 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 Tie-2 and PDGF and TGF-β have shown promising results in regression of neovascularisation and vessel stabilisation. The Tie-2 receptor, similar to VEGF-R, is expressed in the endothelium and has 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 proangiogenic 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 (mAb) against ANG-2 for the treatment of DME.

PDGF is another growth factor that stimulates blood vessel formation, proliferation, and angiogenesis, and might 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 are abnormally formed, leading to their regression [77]. Another example is the development of rinucumab, an IgG4 mAb that targets PDGF-R. These findings suggest biologics inhibiting the PDGF or PDGFR pathway as valid approaches for the treatment of ocular neovascularisation.

Drug combinations to target multiple ligands or receptors are widely used successfully in different areas of medicine, including oncology and infection. In ocular neovascularisation, efforts have been made to design and formulate drug combinations with multiple targets, with several examples in Phase II trials; however, so far, these

have not 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 with aflibercept monotherapy [15,16].

Although the vitreous is an acellular compartment of the eye, there are cellular targets e present in the retinal tissue. Tissue factor (TF) is a surface receptor target for coagulation factor VII that initiates the extrinsic coagulation pathway and, thus, has an important role in retinal neovascularisation [79]. In a normal healthy eye, TF is only expressed in response to inflammation by vascular endothelial cells, monocytes, and macrophages [80]. IVI of anti-TF mAb resulted in a reduction of choroidal neovascularisation (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 CNV [81–83]. ICON-1 is an Fc-fusion protein comprising 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 with 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 therapeutic role in inhibiting CNV in patients with AMD. In general, integrins are transmembrane proteins that bind to extracellular matrix (ECM) proteins, such as laminin, fibronectin, and collagen. Integrin α5β1 is a fibronectin receptor involved in endothelial cell migration and proliferation [86]. Volociximab is a mAb that binds to fibronectin to inhibit its binding to integrin α5β1. 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 mAb (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-ß, IL-17A and IL-23) [90], could have an important role in the progression of neovascular and inflammatory diseases. Although targets to treat inflammation have begun to emerge, most investigations have been conducted by administering the antibodies parenterally rather than by IVI because intravitreal formulations have not yet been developed. Adalimumab is a fully human mAb 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 IL-6R and is approved for the treatment of rheumatoid arthritis, and is currently in Phase II trials for the treatment of refractory Behçet's uveitis. Elevated concentrations of IL-6 have been detected in the vitreous of patients with posterior uveitis [92,93]. Safety and efficacy of another anti-IL-6R antibody, sarilumab, is being evaluated in Phase II trials for posterior segment non-infectious uveitis. Satralizumab, an anti-IL6R antibody, was recently developed for another inflammatory-related disease, neuromyelitis optica spectrum disorder, which is a rare neurological brain condition caused by inflammation in the optic nerve.

Canakinumab and gevokizumab are two antibodies targeting IL-1ß. Canakinumab is approved for treatment of two forms of cryopyrin-associated periodic syndrome (CAPS), and an intravenous formulation completed Phase II trials in July 2020for Behçet'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 (secukinumab) and anti-IL-23 (ustekinumab) antibodies. Whereas secukinumab failed to progress to Phase III trials [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 because muscles and fatty tissues behind the eye become inflamed [98].

Antibodies targeting anti-inflammatory cytokines are given in high does (e.g., 5 mg/kg) because they are systemically administrated by the parenteral route. High doses are necessary to achieve some biodistribution within the eye. Intravitreal dosage forms have not been developed and, thus, 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. Given that intravitreal doses are low relative to parenteral doses, there would be less systemically associated adverse effects.

Dual therapeutic targeting

Faricimab (Figure 1a) is an IgG antibody with the ability to bind two therapeutic targets. It comprises one Fab with specificity for VEGF and another Fab with specificity for ANG-2, and is in Phase III trials for the treatment of wet-AMD [30] and DME [29]. By 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 because of their 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 because 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 the efficacy and pharmacokinetics (PK) of faricimab using an 8-week dosing interval with aflibercept for both wet-AMD and DME treatment [29,78]. Given the need to avoid ocular toxicity and develop more efficacious drugs, faricimab appears to demonstrate a favourable safety profile as a bispecific. 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 of a dual-acting antibody with two therapeutic targets is 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 soluble VEGF and TNF-α receptors, which are fused to a Fc IgG region. A study showed that, compared with two control monospecific (anti-VEGF aflibercept and anti-TNFα etanercept) therapies, valpha has the potential to increase treatment effectiveness because of its dual-targeting approach and favourable PK profile [101]. Valpha also 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. Although 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 and/or systemic cytotoxicity, as reviewed in the 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 could 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 who 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 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 extrascleral flange with a self-sealing septum designed to allow access to the reservoir to remove and replenish drug in a clinical setting using aseptic techniques. Although this strategy avoids the need for IVI, 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 high-molecular-weight molecule with 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 with the influence of the size of the therapeutic protein in solution (i.e., hydrodynamic radius) [116]. Given that the vitreous often becomes less viscous as we age, 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 principle 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 theory be achieved by using 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) or collagen] (Table 2) or 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 might be higher in some disease conditions, such as DR [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. Although targeting albumin in circulation is well known and is a clinically proven strategy [126–128], utilising albumin in the eye might be limited if the drug is also not required systemically after clearing from the eye.

Researchers from Novartis described a bispecific molecule comprising an anti-VEGF Fab fused with the HA-binding component derived from hyaladherin [122]. Results showed that HA binding anti-VEGF adducts displayed approximately three to fourfold longer half lives in rabbit and monkey eyes compared with non-HA binding controls. Inhibition of VEGF-induced vascular leak was also three to four times longer in animal models with the HA-binding bispecifics.

Another example was described by Roche [129], which reported that the preparation of a recombinant fusion protein (peptide linker) with the first binding site (Fab or scFv) had therapeutic action targeting VEGF, and the second binding site specifically bound 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 with the therapeutic Fab without the affinity binding moiety.

Targeting heparan sulfate proteoglycans (HSPGs) has also been suggested as another affinity target to prepare a dual-acting molecule [130]. HSPGs are cell surface glycoproteins of heparan sulfate found on the 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' molecules 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 RPE cells [132]; ~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 recently established a correlation between *in vitro* binding and *in vivo* PK [140]. Drugs can bind to melanin, altering its pharmacologic and PK profiles [141] by forming a reservoir to prolong residence time [142,143]. Intravitreally administered low-molecular-weight molecules that bind to melanin have been reported [138,144,145], namely ß-blockers, celecoxib, and chloroquine. Melanin binding is more pronounced with lipophilic than with lipophobic drugs (e.g., beta-blockers) [146–148].

The duration of action of a drug can also be extended by developing an exogenously administered binding target (or anchor). Shoichet and coworkers explored the affinity between a protein and a hydrogel [121,123,149–152]. In a 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 (HA 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]. Following IVI 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 because of insufficient protein being present at 7 days after IVI [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) have elements of the IgG format. Non-IgG formats referred to earlier 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] might also have potential intraocular applications. Other non-IgG formats [such as nanobodies, Diabodies, bi-specific T-cell engagers (BiTEs), and dual-affinity retargeting antibodies (DARTs)] that have been developed for use in oncology, might not be ideal for systemic use because of suboptimal clearance rates from the blood compartment. However, these might potentially emerge for intraocular use following further development.

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

A bispecific DARPin targeting VEGF and PDGF is currently in preclinical development for ocular diseases [158] and has not yet entered clinical trials. In this molecule, two different DARPins are linked via peptide linkage (Figure 1b). Nanobodies are derived from camelids, including camels, llamas, and alpacas [159]. They comprise heavy chain variable regions and have molecular weights as low as 13 kDa. Nanobodies share several advantages with DARPins that might be important for their intraocular use, 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 currently being developed for ocular diseases. However, an interesting bispecific nanobody, called BI 836880, which 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 comprising two scFvs and have a molecular weight of ~50 kDa. They are manufactured with peptides linking two scFvs derived from different monospecific mAbs [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 in the high concentration formulations [163] required for ocular application. In addition, there are general concerns regarding the stability, toxicity, and immunogenicity caused by peptide linkers for diabodies and BiTE molecules, which might 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 investigate 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 PK 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. A monospecific $F(ab)_2$-like format, a Fab-PEG-Fab molecule (FpF), was recently synthesized using a recombinant-chemical approach. The FpF molecule shows many similarities to an IgG molecule (e.g., solution size and binding affinity) and 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 constants (k_{off}) compared with 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 could be a viable approach to increase efficacy within ocular tissue. Fc-fusion mimetics called receptor-PEG-receptors (RpRs) have also been prepared with similar binding properties, and bispecific FpFs (Figure 2b) are currently being designed for intraocular applications [171].

Concluding remarks and future prospects

As with any therapeutic, target selection is a first crucial 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 might 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, based purely on spatial temporal relationships, there might be more opportunities than anticipated 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, and all molecular formats will require thorough evaluation for intraocular use.

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Author biographies

Matthew Collins

Matthew Collins is studying for a PhD at the University of East London, working on a project to develop hybrid strategies to generate bispecific antibody mimetics. He earnt his MSc in clinical drug development from Queen Mary University of London in 2017.

Sahar Awwad

Sahar Awwad is a postdoctoral research fellow at UCL, School of Pharmacy and UCL, Institute of Ophthalmology. Her current research projects are widely based on (i) drug delivery; (ii) ocular pharmaceutics, pharmacokinetics and biodistribution studies; and (iii) protein production and modification; and she has extensive knowledge of protein characterisation, modification and conjugation. She has also developed the PK-Eye, an innovative two-compartment *in vitro* model of the human eye to aid in the evaluation of ocular formulations. This led to the formation of Optceutics Ltd, a spin-out company that utilises the PK-Eye with UK-US investment.

Hanieh Khalili

Hanieh Khalili was awarded a PhD in biotherapeutics in 2012 from University College of London, School of Pharmacy. She continued for additional 4 years as postdoctoral research fellow at UCL School of Pharmacy and Institute of Ophthalmology working on the development and formulation of novel antibody mimetics for ocular inflammation. She is currently a lecturer in pharmaceutics at University of East London, School of Health, Sport and Bioscience. Her research focuses on the generation and formulation of bispecific antibody mimetics using recombinant-chemical approaches. Her expertise is to characterise and analyse the binding affinity and thermodynamic properties of modified proteins using protein–protein interaction assays, such as surface plasmon resonance and isothermal titration calorimetry.

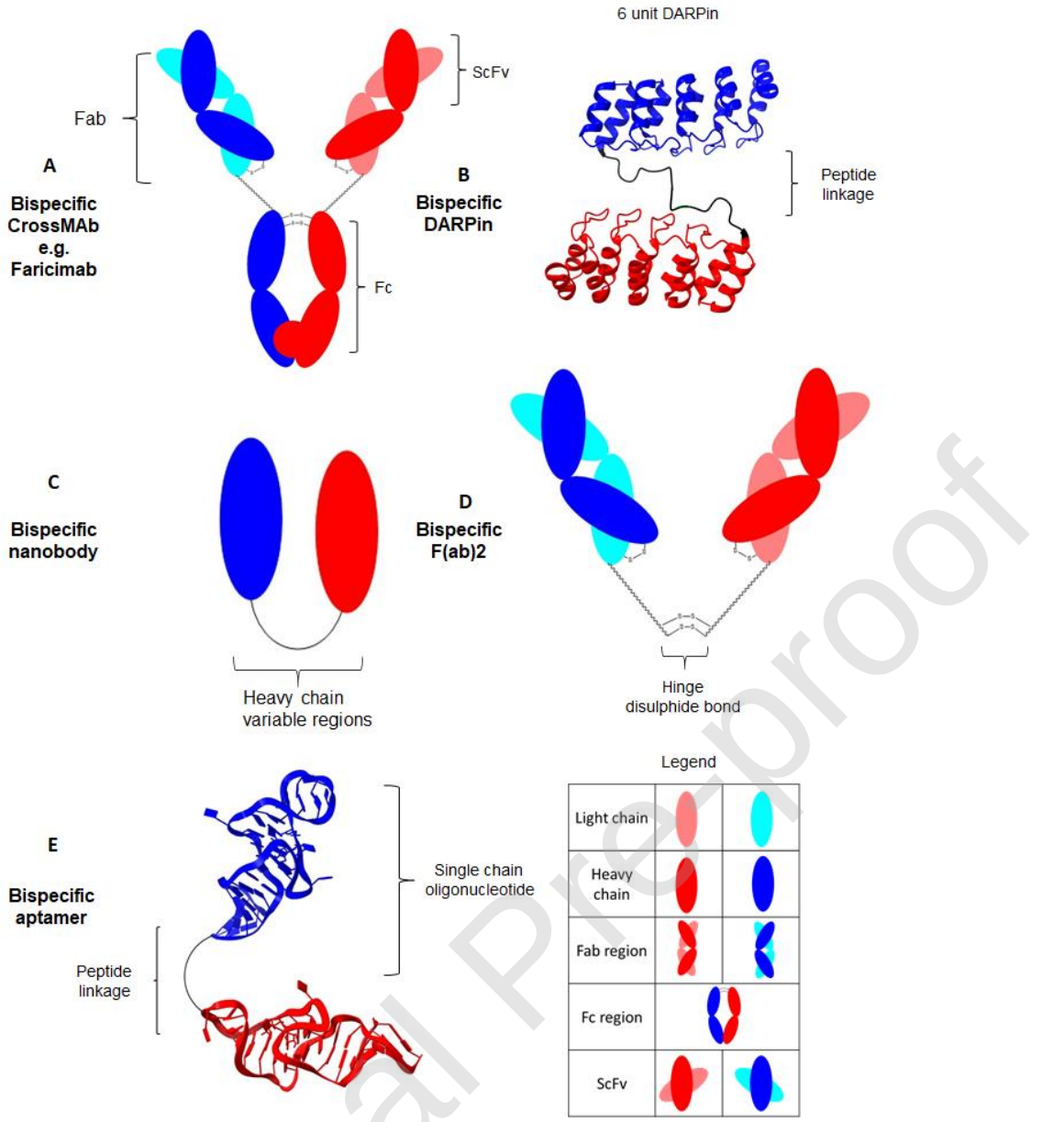

Figure 1. Structures of five bispecific antibodies [immunoglobulin (Ig)G and non-IgG formats] for ocular indications. **(a)** A bispecific antibody produced using CrossMAb technology. **(b)** A bispecific designed ankyrin repeat protein (DARPin) containing two DARPins linked by a peptide linker [adapted from Protein Data Bank (PDB) 500U]. **(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 disulfide bond. **(e)** A bispecific aptamer molecule containing two oligonucleotide aptamers linked together via a peptide linkage (adapted from PDB 2AU4).

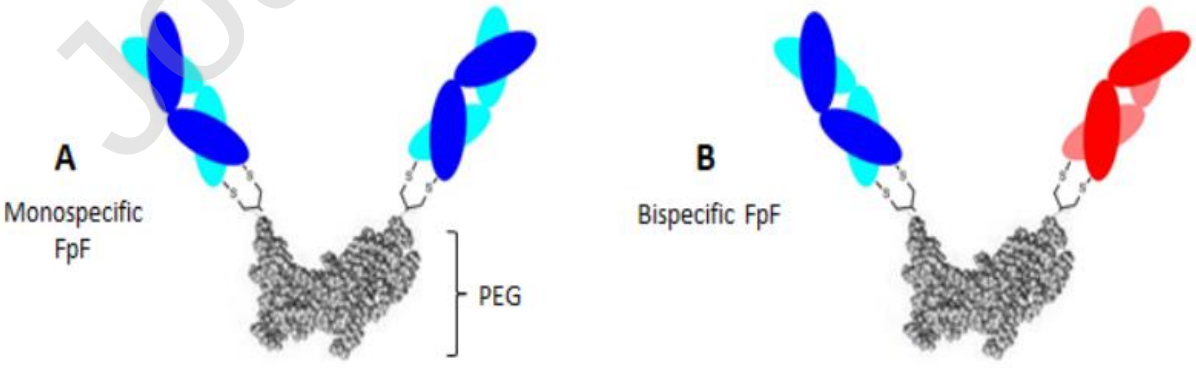

Figure 2. Structures of monospecific and bispecific Fab-PEG-Fab molecule molecules. **(a)** A monospecific FpF in which two identical Fabs are covalently bound

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.

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

Therapeutic target	Name of drug	Format	Clinical progress	Clinical dose (mg)	Molar dose per injection	Company	Refs/Clinical trial no.
Neovascular ligand							
VEGF	*Abicipar Pegol (34 kDa)*	*PEGylated DARPin*	*Phase III (rejected by FDA, July 2020)*	*2 mg (50 ml IVI)*	*3–4*	*Molecular Partners/Allergan*	*NCT02462486; NCT02462928 [63]*
	KSI-301 (950 kDa)	*IgG1 biopolymer conjugate*	*Phase II [4]*	*5 mg (by the weight of antibody) (50 ml IVI)*	*3–4*	*Kodiak*	*NCT04049266*
	OPT-302 (115 kDa)	*Fc-fusion*	*Phase IIb [5]*	*2 mg (50 ml IVI)*	*1*	*Opthea*	*NCT03345082*
	Conbercept (140 kDa)	*Fc-fusion*	*Phase III [6–13]*	*2 mg (50 ml IVI)*	*1*	*Chengdu Kanghong*	*NCT02194634; NCT04296838; NCT01809223; NCT03223714; NCT03108352; NCT03577899; NCT03630952; NCT01436864*
PGDF	*Rinucumab (150 kDa)*	*IgG4*	*Phase II in combination with anti-VEGF drug (no benefit over VEGF monotherapy)*	*3 mg (50 ml IVI)*	*1.0*	*Regeneron*	*[18,172,173]*
ANG-2	*Nesvacumab (150 kDa)*	*IgG1*	*Phase II in combination with anti-VEGF drug (discontinued)*	*6 mg (50 ml IVI)*	*2*	*Regeneron*	*[174]*
VEGF/ANG-2	*Faricimab (150 kDa)*	*Bispecific CrossMab*	*Phase III*	*6 mg (50 ml IVI)*	*2*	*Roche*	*NCT03823287; NCT03823300; NCT03622580; NCT03622593*
Neovascular receptor							
TF	*HI-con1 (115 kDa)*	*Fc-fusion protein*	*Phase II*	*0.5 mg (50 ml IVI)*	*0.3–0.4*	*Iconic Therapeutics*	*NCT03452527; NCT02358889*
Fibronectin receptor	*Volociximab (150 kDa)*	*IgG1*	*Phase I*	*2.5 mg (50 ml IVI)*	*1*	*Ophthotech*	*NCT00782093*
Inflammatory ligand							
TNF-a	*Adalimumab (150 kDa)*	*IgG1*	*Approved for non-infectious uveitis*	*Up to 40 mg (SC)*	*–*	*Abbott*	*[5,91]*
IL-6R	*Tocilizumab (150 kDa)*	*IgG1*	*Phase II for non-infectious uveitis*	*8 mg/kg (IVI); 162 mg (SC)*	*–*	*Roche*	*NCT03554161*
	Sarilumab	*IgG1*	*Phase II*	*200 mg (SC)*	*–*	*Sanofi/Regeneron*	*[175]*
IL-1β	*Canakinumab*	*IgG1*	*Phase II completed in 2019*	*300 mg (IVI)*	*–*	*Novartis*	*NCT02756650*
	Gevokizumab	*IgG1*	*Phase III (failed to meet primary endpoint)*	*60 mg (SC)*	*–*	*XOMA/Novartis*	*[94]*
IL-17A	*Secukimumab*	*IgG1*	*Phase III (failed to meet primary endpoint)*	*Up to 300 mg (SC)*	*–*	*Novartis*	*[96]*
IL-23	*Ustekinumab*	*IgG1*	*Phase II*	*90 mg (SC); up to 520 mg (IVI)*	*–*	*Janssen*	*NCT02648581; NCT02911116*

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

Target combination (therapeutic + affinity)	Name of drug	Format	Group/Company	Refs
VEGF + hyaluronan	*NVS24*	*Fab + HA-binding domain of TSG-6*	*Novartis*	*[122]*
VEGF + HSPGs	*Sticky-trap*	*Fc-fusion (VEGF trap fused with HBDs)*	*Michael et al.*	*[131]*
VEGF + Collagen II	*Undisclosed*	*Fusion protein (scFv fused with Fab)*	*Roche*	*[129]*

[a]*Abbreviation: TfR, transferrin receptor.*