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Prolonged intraocular residence and retinal tissue distribution of a fourth-generation compstatin-based C3 inhibitor in non-human primates

Sarah Hughes[a,1], Justin Gumas[a,1], Rebecca Lee[a], Merita Rumano[a], Nadja Berger[a], Avneesh Kumar Gautam[a], Georgia Sfyroera[a], Anna Lorena Chan[b], Gopalan Gnanaguru[c,d], Kip M. Connor[c,d], Benjamin J. Kim[e], Joshua L. Dunaief[e], Daniel Ricklin[f], George Hajishengallis[g], Despina Yancopoulou[h], Edimara S. Reis[a], Dimitrios C. Mastellos[i,*], John D. Lambris[a,*]

[a] Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[b] Jose R. Reyes Memorial Medical Center, Manila, Philippines
[c] Angiogenesis Laboratory, Department of Ophthalmology, Massachusetts Eye & Ear Infirmary, Boston, MA, USA
[d] Department of Ophthalmology, Harvard Medical School, Boston, MA, United States
[e] Department of Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA, USA
[f] Department of Pharmaceutical Sciences, University of Basel, Switzerland
[g] Department of Basic and Translational Sciences, Penn Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA
[h] Amyndas Pharmaceuticals, Glyfada, Greece
[i] National Center for Scientific Research 'Demokritos', Athens, Greece

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ABSTRACT

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss among the elderly population. Genetic studies in susceptible individuals have linked this ocular disease to deregulated complement activity that culminates in increased C3 turnover, retinal inflammation and photoreceptor loss. Therapeutic targeting of C3 has therefore emerged as a promising strategy for broadly intercepting the detrimental proinflammatory consequences of complement activation in the retinal tissue. In this regard, a PEGylated second-generation derivative of the compstatin family of C3-targeted inhibitors is currently in late-stage clinical development as a treatment option for geographic atrophy, an advanced form of AMD which lacks approved therapy. While efficacy has been strongly suggested in phase 2 clinical trials, crucial aspects still remain to be defined with regard to the ocular bioavailability, tissue distribution and residence, and dosing frequency of such inhibitors in AMD patients. Here we report the intraocular distribution and pharmacokinetic profile of the fourth-generation compstatin analog, Cp40-KKK in cynomolgus monkeys following a single intravitreal injection. Using a sensitive surface plasmon resonance (SPR)-based competition assay and ELISA, we have quantified both the amount of inhibitor and the concentration of C3 retained in the vitreous of Cp40-KKK-injected animals. Cp40-KKK displays prolonged intraocular residence, being detected at C3-saturating levels for over 3 months after a single intravitreal injection. Moreover, we have probed the distribution of Cp40-KKK within the ocular tissue by means of immunohistochemistry and highly specific anti-Cp40-KKK antibodies. Both C3 and Cp40-KKK were detected in the retinal tissue of inhibitor-injected animals, with prominent co-localization in the choroid one-month post intravitreal injection. These results attest to the high retinal tissue penetrance and target-driven distribution of Cp40-KKK. Given its subnanomolar binding affinity and prolonged ocular residence, Cp40-KKK constitutes a promising drug candidate for ocular pathologies underpinned by deregulated C3 activation.

1. Introduction

AMD is a prevalent ocular disease with complement-driven pathophysiology that can lead to irreversible vision loss in affected elderly individuals [1]. It progresses from its early form, marked by accumulation of lipid and protein-rich deposits (drusen) within Bruch's membrane and resulting sub-retinal inflammation, to the advanced dry form (geographic atrophy, GA) in which there is loss of choroidal vessels,

* Corresponding authors.
E-mail addresses: mastellos@rrp.demokritos.gr (D.C. Mastellos), lambris@pennmedicine.upenn.edu (J.D. Lambris).
[1] These authors contributed equally to this study.

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retinal pigment epithelium (RPE), and photoreceptors [1]. Dry AMD can evolve into a wet (neovascular) form characterized by aberrant neovascularization with vessel leakage and further inflammatory damage often leading to retinal atrophy and vision loss [1,2]. Advanced dry AMD (GA) still lacks approved treatment, while the current standard of treatment for wet AMD is locally administered anti-vascular endothelial growth factor (VEGF) therapy [1].

Over a decade ago, the discovery that a common genetic variant (p.Y402H) of the complement regulator factor H (FH) predisposes individuals to AMD drastically reshaped our perception of how the complement system modulates disease progression in AMD and other retinal diseases driven by deregulated complement activation [3,4]. Extensive genetic studies have identified several risk-associated common or rare genetic variants in different components and regulatory proteins of the alternative complement pathway (AP) [1,5]. Whereas the precise role of complement deregulation in the pathophysiology of AMD still remains ill-defined, there is compelling evidence that a deregulated AP response leading to increased C3 turnover and retinal inflammation is integrally involved in the early stages of this disease [6]. This growing appreciation of complement's involvement in AMD pathology has ignited efforts to target this system therapeutically by tapping into various druggable targets of the cascade [6,7].

Given the disease-exacerbating role of the AP, initial efforts to develop therapeutics for treating GA focused on factor D (FD), the rate-limiting protease of this pathway, responsible for the formation of the AP C3 convertase. However, the failure of the FD-targeting mAb, lampalizumab (Genentech/Roche) to meet its primary endpoint in two multicenter phase III trials signified a setback in the clinical development of FD inhibitors for AMD [8]. At the same time these trials raised awareness about possible gaps in the biology of FD's involvement in AMD and phenomena that may curb clinical efficacy, such as, reduced drug bioavailability, insufficient tissue penetration or potential FD bypass pathways that may become operative in a prolonged therapeutic regimen. Despite this clinical setback, the pharmaceutical industry has rekindled its efforts to develop ocular therapeutics focused on alternative targets, including factor B (FB) and C5 inhibitors [7,9]. Interestingly, Zimura/avacincaptad pegol, a PEGylated anti-C5 aptamer developed by Iveric (formerly Opthotech) has shown promising results in a phase IIb trial in GA patients with significant reduction in GA lesion size [10], thus expanding the toolbox of drug candidates for AMD.

While these approaches have shown early clinical promise, the therapeutic targeting of C3 activation has gained considerable traction in recent years as a more comprehensive strategy whereby all pathways are broadly inhibited at the level of C3, regardless of initiating triggers or downstream effector mechanisms [9]. C3 inhibition can single-handedly afford therapeutic coverage against multiple pathogenic drivers in AMD by preventing generation of both C3 and C5-derived fragments that modulate phagocytic cell recruitment, oxidative tissue damage, inflammatory cell activation and cytolytic activity via membrane attack complex (MAC) assembly [11,12].

In this respect, peptidic C3 inhibitors of the compstatin family have entered clinical development as promising ocular therapeutics [6,7]. 4(1MeW)7W/POT-4, a second-generation compstatin analog was initially evaluated in a phase I study in wet AMD patients showing good safety and tolerability (Potentia/Alcon) [13]. Despite the lack of clinical efficacy in phase II trials in wet AMD, likely because of insufficient dosing [14], initial studies with POT-4 propelled the development of its PEGylated version, APL-2/pegcetacoplan (Apellis). This C3 therapeutic has recently completed a phase II trial in GA patients having shown safety and therapeutic efficacy in terms of reducing GA lesion size independently of genetic variants that can skew GA progression [15]. Pegcetacoplan is currently being evaluated in two multi-center phase III studies in GA patients in monthly or bimonthly dosing regimens [16]. While PEGylation has likely increased the intraocular retention of APL-2, as compared to POT-4, the presence of a fraction of patients with wet AMD conversions in the phase II trial raises the possibility that at high PEG burdens, PEG–triggered choroidal neovascularization (CNV) or other mechanisms could modulate the clinical outcome [17,18]. The incidence of a similar CNV conversion rate in GA patients receiving the PEGylated therapeutic Zimura provides further evidence for this scenario, suggesting that alternative PEG-free formulations should be explored for delivering complement inhibitors intraocularly [19] [10].

The development of third and fourth-generation non-PEGylated compstatins with improved target affinity, solubility and favourable pharmacokinetic (PK) profiles may confer benefits in terms of improved efficacy, reduced dosing frequency and lower risk for PEG-related adverse events in AMD patients [7,13,20]. AMY-101 (Amyndas), a C3 therapeutic based on the third-generation compstatin analog Cp40 [21], is currently evaluated in phase IIa trials in patients with periodontal disease, and is clinically developed for a spectrum of renal and hemolytic indications, further illustrating the clinical feasibility of this approach [22–25]. Here we report the intraocular pharmacokinetic profile and retinal tissue distribution of the fourth-generation compstatin analog, Cp40-KKK [20]. Its prolonged intraocular residence at C3-saturating levels, for over 3 months after a single intravitreal (IVT) injection, makes it a suitable drug candidate for ocular indications associated with chronic C3 dysregulation.

2. Materials and methods

2.1. Inhibitors and reagents

The compstatin analog Cp40 (dTyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH_2, 1.8 kDa) and two derivatives containing either two or three lysine residues at the C-terminus (Cp40-KK and Cp40-KKK) was synthesized by solid-phase peptide synthesis, cyclized by disulfide bridge formation, and purified as previously described [20,21]. Plasma-purified human C3 was purchased from Complement Techology (Tyler, TX). Pooled EDTA-human plasma was purchased from Innovative Research Inc. (Michigan, USA).

2.2. Animals

All animal studies were performed in accordance with animal welfare laws and regulations, as approved by the Institutional Animal Care and Use Committee (IACUC). This study consisted of two separate animal protocols. The animals used in the first protocol (six) were housed at the Primate Center, Hamamatsu Pharma Research, Inc. (Hamamatsu, Shizuoka, Japan) while the animals used in the second protocol (nine) (see details below) were housed at the Simian Conservation Breeding and Research Center (SICONBREC, Makati City, Philippines). In the first protocol, cynomolgus monkeys (*Macaca fascicularis*) were treated with one dose of 500 μg of Cp40-KK, Cp40-KKK, mPEG(3k)-Cp40 or mPEG(1k)-Cp40 peptides via IVT injection (3 eyes injected per treatment group). For IVT injection, the animals were anesthetized with a combination of ketamine (15–25 mg/kg, i.m.) and xylazine (2 mg/kg, i.m.) and the eye was cleaned with povidone-iodine solution. After application of proparacaine ophthalmic solution to the cornea as a local anesthetic, Cp40-KK or Cp40-KKK was administered to the animals by IVT injection into either the right or left eye of each animal. Immediately following each injection, a single topical dose of 0.5% moxifloxacin was administered for antibiotic treatment. For intraocular pharmacokinetic analysis, vitreous samples (about 50 μL each) were sequentially collected from the same animal at 14, 28, 42, 56, 73, and 90 days post injection. Samples were collected on ice and stored in a deep freezer (−79.3 to −68.5 °C) until further analysis. In the second protocol, a dose titration study was conducted in cynomolgus monkeys with IVT administration of 25, 100, or 500 μg Cp40-KKK. In order to assess the retinal tissue distribution of Cp40-KKK, the treated non-human primates (NHPs) were euthanized one month following the injection of inhibitor.

For immunohistochemistry, both right and left eyes of cynomolgus

monkeys were collected, fixed in 4% paraformaldehyde solution in PBS and stored at 4 °C until dissection. The eyecup was carefully dissected into two halves; half of the eyecup containing the optic nerve head was cryopreserved overnight in 30% sucrose (prepared in PBS) at 4 °C. The following day, the sample was embedded in OCT, frozen in isopropanol (precooled in dry ice), and the tissue was sectioned at 12-μm thick sections and stored at −80 °C.

2.3. SPR-based competition assay for quantification of Cp40-KK and Cp40-KKK in NHP vitreous

An SPR-based competition assay for quantification of the Cp40 analogs bearing lysine extensions at the C-terminus was performed on NHP eye vitreous samples using a Biacore 3000 instrument (GE Healthcare, Piscataway Township, NJ) at 25 °C. In brief, Cp40-KKK was covalently immobilized on a CM5 sensor chip by standard amine coupling, and an untreated flow cell was used as reference for subtraction of bulk refractive index changes. Non-specific binding was reduced by adding dextran sulfate (1 mg/ml) to the running buffer (50 mM sodium phosphate, 100 mM NaCl, 0.05% Tween-20, 10 mM EDTA, 0.02% NaN_3, pH 7.4). All samples were diluted in running buffer. For quantitation of CP40-KK or Cp40-KKK in different vitreous samples, those samples were initially diluted 1:3, however in later experiments dilutions were adjusted according to projected concentrations (up to 1:100). Next these samples were heat-inactivated for 5 min at 95 °C, which allows for the release of all C3-bound Cp40-KK or Cp40-KKK molecules in solution. After a cooling phase, samples were subjected to centrifugation (10 min at 14,000 ×*g*) and the supernatant was mixed with a fixed concentration of human C3 (82 nM or 15.3 μg/ml), which served as a detection agent in this competition assay. Pooled EDTA-human plasma served as the source of human C3. To this end, supernatants were spiked with 67-fold diluted pooled human plasma containing the desired concentration of C3. Different concentrations of CP40-KK or Cp40-KKK in the test samples will compete with chip-bound Cp40 analogs for binding to spiked C3 (from human plasma) and produce an inversely proportionate signal with Cp40-KK or Cp40-KKK concentrations.

Different samples were injected for 2 min at a flow rate of 20 μl/min; after each run the sensor chip surface was regenerated by injection of 0.5% SDS for 1 min followed by one injection of 50 mM glycine buffer, pH 8.5, for 30s. Data was analyzed in Scrubber software by double referencing, and the binding signal was calculated from the average response, between 30s–50s after the injection's end. Standard curves for each experiment were generated using the same injection sequence for different concentrations of Cp40-KK or Cp40-KKK (225 nM - 4 nM). Control experiments were conducted to determine (a) the heat stability of Cp40-KK or Cp40-KKK by testing their binding to C3 after incubation at 95 °C for different time lengths; (b) the specificity of Cp40-KK or Cp40-KKK for binding to C3 by injecting 1:50-diluted, C3-depleted human plasma in presence or absence of 120 nM C3; and (c) the robustness of this method by generating standard curves in plasma samples from different donors and in buffer alone.

2.4. Sandwich ELISA for quantification of intravitreal C3

The quantification of intravitreal C3 was performed with an in-house developed sandwich enzyme-linked immunosorbent assay (ELISA). For this purpose, an anti-C3b/iC3b monoclonal antibody (D131-45A-8E11; generously provided by Dr. Ronald Taylor, University of Virginia) and an HRP-conjugated goat anti-human C3 antibody (ICN Cappel, Costa Mesa, CA) were used as capture and detection antibodies, respectively. It was previously established that the capturing and detection antibodies cross-react with human and NHP C3 [26]. In brief, microtiter wells (NUNC) were coated with 50 μl of 1 μg/ml capturing antibody in PBS and incubated at 25 °C for 2 h. The wells were blocked with 200 μL of 1% BSA in PBS for 1 h. Plates were washed three times with PBS containing 0.005% Tween-20. Dilutions of purified C3 were made ranging from 1.87 μg/ml to 0.23 pg/mL, and test samples were diluted in sample diluent (1% BSA) and incubated at 25 °C for 1 h. After washing with PBS/Tween, the HRP-conjugated detection antibody (1:1000; ICN Cappel) was added and incubated at 25 °C for 1 h. The plate was washed thoroughly, and TMB substrate was added to each well and incubated at 25 °C for 10 min in the absence of light. Finally, to stop the reaction, 0.1 N H_2SO_4 was added and the plate was read at 450 nm using a VICTOR multilabel plate reader (PerkinElmer, Waltham, MA).

2.5. Anti-Cp40 antibody generation

The compstatin analog Cp40 was conjugated to keyhole limpet hemocyanin (KLH; Sigma-Aldrich) using established protocols. The resulting Cp40-KLH conjugate was used in the immunization of rabbits (performed at Cocalico Biologicals). Rabbit sera were collected and tested for reactivity to various Cp40-based compstatin analogs, including Cp40-KKK, by ELISA. Rabbit antisera testing positive for antibodies against Cp40-KKK were purified by means of affinity chromatography on a HiTrap NHS-Activated HP affinity column (GE Life Sciences). The Cp40-KK peptide was coupled to the affinity column using the manufacturer's instructions. The presence of affinity-purified antibodies was confirmed by SDS-PAGE and direct ELISA.

2.6. Immunohistochemistry for C3/Cp40-KKK detection in retinal tissue

Immunofluorescent staining of NHP retinal tissue sections was performed using standard methods. Briefly, CryoStat-preserved retinal sections were thawed at room temperature, air-dried for 30 min, and washed 3 times in PBS for 10 min each time. Following washes, the sections were permeabilized in PBS containing 0.1% Triton X-100 for 10 min at room temperature and blocked in background buster solution (Innovex Biosciences) for 1 h at room temperature. The sections were then incubated with both goat anti-human C3 (Invitrogen, PA1–29715, 1:200 dilution) and rabbit anti-Cp40-KKK antibody (generated as described above, at a final concentration of 2 μg/ml) overnight at 4 °C. The following day, the sections were washed 3 times in PBS for 10 min each time before detection. For the C3 staining, washed sections were incubated with either Alexa Fluor 647-labeled chicken anti-goat IgG (H + L; Invitrogen) at room temperature for 1 h. For Cp40-KKK staining, the incubation was performed with goat anti-rabbit-HRP (Thermo Fisher) followed by Alexa Fluor™ 555 Tyramide (Thermo Fisher, B40923) for 10 min and reaction stop solution according to the instructions of the manufacturer. All sections were washed three times in PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI, final concentration of 1 μg/ml) for 10 min. After three washes, the sections were incubated for 30 s with trueblack® autofluorescence quenching agent (Biotium, Inc). After quenching step, the sections were washed twice in PBS for 5 min each time and then coversliped using FluoRo-GEL (17985–10, EMS) and imaged using Nikon Eclipse Ti2 Confocal Microscope (200× magnification).

3. Results and discussion

3.1. Prolonged residence time of compstatin analogs Cp40-KK and Cp40-KKK in the vitreous of non-human primates

The advancement of the PEGylated C3-targeted therapeutic APL-2/pegcetacoplan (Apellis) to phase III trials in diseases of the hemolytic and ocular spectrum (i.e., PNH and GA/AMD) has marked an important milestone in the path towards clinical C3 inhibition [6,27,28]. Clinical results are now validating the safety and efficacy of this long-debated targeting strategy, thereby eliminating the purported risk of compromised pathogen immunosurveillance during chronic anti-C3 treatment [7]. While a second-generation compstatin-based C3 inhibitor has

entered clinical development as a treatment option for retinal diseases (AMD), there are still unmet translational challenges in terms of characterizing the intraocular pharmacokinetic profile of these peptide inhibitors and monitoring in situ activity and retinal tissue distribution across the time window of therapeutic intervention. The recently completed phase II trials of APL-2 in GA patients have prompted consideration of alternative IVT formulations of these C3 inhibitors that could leverage a further improvement of the drug dosing window in AMD patients [17]. The recent disclosure of fourth-generation compstatin derivatives with markedly improved inhibitory potency over APL-2, enhanced solubility and improved pharmacokinetic profiles for chronic administration has galvanized efforts to evaluate these novel C3 therapeutics in the ocular space [20]. A crucial first step towards their clinical translation is the thorough characterization of the ocular PK profile and residence time of the lysine-modified Cp40 analogs, Cp40-KK and Cp40-KKK, in the ocular compartment using an appropriate preclinical model.

The scarcity of material that can be recovered from the eye vitreous, along with the species specificity of compstatins that dictates the selection of nonhuman primates as the only suitable model for PK/PD profiling, both necessitated the development of sensitive methods for reliably quantitating the concentration, activity and tissue distribution of these C3 inhibitors in the ocular compartment. To facilitate intraocular PK measurements of Cp40-KK and Cp40-KKK in cynomolgus monkeys we first developed a sensitive detection platform that relies on a fully customized SPR-based competition assay that can quantify trace amounts of Cp40 analogs in vitreous or plasma samples. To this end, Cp40-KKK was immobilized on an SPR sensor chip and served as 'bait' for the real-time monitoring of the competitive binding of vitreous-derived Cp40-KK or Cp40-KKK to a standard amount of human C3 used as reference (Fig. 1, *panel A*). Although the coupling of Cp40-KKK to the chip may occur at any of the four primary amino groups of the molecule (i.e. N-terminal D-Tyr or any of the three C-terminal Lys), this does not affect the detection principle as the binding of C3 primarily occurs to the cyclic part of the peptide [13]. As a sample preparation step, heat inactivation of the diluted vitreous sample was performed for dissociating the compstatin molecules from their target C3 (i.e., C3/C3b/C3c). Subsequently the heat-inactivated vitreous sample was mixed with a calibrated amount of pooled human plasma, serving as a source of C3, and the mixture was flown over the Cp40-KKK CM5 sensor chip. In this setup the total Cp40 analog molecules (both unbound and released from the target-bound complexes) compete with the sensor-immobilized Cp40-KKK for binding to free C3. By directly measuring the unbound fraction of a constant pool of C3, changes of the SPR signal accurately reflect the total amount of the Cp40 analogs (i.e., Cp40-KK or Cp40-KKK) in the vitreous sample. Quantification was performed using a standard curve of serially diluted known peptide concentrations (Fig. 1, *panel A*). There are notable advantages of this SPR-based detection method: (a) it enables inhibitor quantification in the vitreous even at trace amounts, with a low limit of detection (LOD) that exceeds the sensitivity of mass spectrometric (MS) analysis (LOD of 0.01 μg/ml and 0.18 μg/ml, respectively) [29]; (b) it features a wider detection range at the lower concentration range when compared to the MS method (0.01–0.3 μg/ml of Cp40-KKK and 0.18–3.58 μg/ml, respectively); and (c) it enables the recovery of the C3 inhibitor in a fully active conformation following heat dissociation, as demonstrated by its SPR-monitored binding kinetics with human C3.

As shown in Fig. 1 (*panel B*), a single IVT injection of 0.5 mg Cp40-KKK in cynomolgus monkeys resulted in sustained presence of this inhibitor in the eye vitreous for a period of more than 90 days post injection. A similar ocular PK profile was obtained in the case of the Cp40-based analog, Cp40-KK (Fig. 1B). During the first month post injection the intravitreal levels of both Cp40-KK and Cp40-KKK remained constant at approximately 200 nM and then slowly decreased reaching a concentration of 100–120 nM by the end of month 3. Of note, both compstatin analogs (Cp40-KK, Cp40-KKK) not only displayed prolonged ocular residence in injected animals, but also remained detectable intraocularly at levels that exceed the average local concentration of C3 by almost 100-fold. Using a sensitive ELISA that captures the total amount of C3 protein present in the vitreous, we determined an average concentration of intravitreal C3 ranging from approximately 0.1–2 nM, which is far below the reported concentration of intravitreal C3 (average C3 concentration: 70 ± 68 nM), according to previous measurements of complement proteins in human vitreous samples [30]. This result was confirmed by western blot analysis of the same vitreous samples using a polyclonal anti-C3c antibody (data not shown). The successive sampling of vitreous humor from the same animal enabled a more accurate determination of the PK profile of the lysine-modified Cp40 analogs. Fig. 1B illustrates the averaged PK profiles of Cp40-KKK and Cp40-KK from a representative experiment where the same amount of inhibitor was injected intravitrealy in 3 separate eyes and monitored in triplicate by SPR-based quantification over a period of 90 days.

3.2. Tissue penetration profile of Cp40-KKK in the retina of non-human primates

While clinical efficacy in reducing the growth of GA lesions has been strongly suggested in a phase 2 trial of the second-generation compstatin derivative APL-2, the tissue distribution profile of C3 inhibitors (i.e., compstatins) in the retina remains to be defined. To gain insight into the tissue distribution of Cp40-KKK in the retina of injected cynomolgus monkeys we developed affinity purified polyclonal anti-Cp40 antibodies and performed immunofluorescent staining in frozen retina sections from Cp40-KKK-treated animals. While C3 staining was predominantly localized to Bruch's membrane and the underlying choroid vasculature of injected NHP eyes, prominent staining for Cp40-KKK was mainly detected in the choriocapillaris, with the C3 inhibitor apparently co-localizing with structural elements of the choroidal microvascular network (Fig. 2) The increased staining for Cp40-KKK in the choriocapillaris cannot rule out the possibility that the compound may have been distributed to other retinal layers in trace amounts that cannot be visualized due to the limited sensitivity of this labeling method. The immunolocalization of the Cp40 analog in the choroid provides evidence that it penetrated the retina and RPE after intravitreal injection. This unique tissue distribution pattern, together with the prolonged residence of Cp40-KKK in the vitreous at levels exceeding its target's concentration, may also imply a continuous redistribution of the compound between different ocular compartments in order to achieve equilibrium over time. The possibility that more compound may steadily replenish the vitreous compartment from a possible depot in the C3-enriched choroid, or via another yet unidentified route, cannot be ruled out. The co-localized staining for both C3 and Cp40-KKK in the choroid indicates that Cp40-KKK not only efficiently penetrates the retinal tissue, but also follows a target-driven tissue distribution profile which is highly dependent on the location of C3 stores or deposits (Fig. 2). The presence of prominent C3 staining in these retinal sections might be attributed to local C3 synthesis by RPE or choroidal endothelial cells, although the contribution of plasma C3 stores entering the tissue through the choroidal blood supply cannot be ruled out. Notably, a similar tissue localization profile was observed after the IVT administration of the Cp40-KK peptide in NHP eyes (data not shown). The exact mechanism by which Cp40 analogs 'home' in different ocular compartments after intravitreal delivery has yet to be fully defined and warrants further investigation.

The prolonged ocular residence of Cp40-KKK appears to be unique to its structure and may be attributed to a tissue-specific mechanism exploiting the presence of the hydrophilic tandem Lys repeat at the C-terminus of the compound. This is further supported by observations that while mini-PEGylated Cp40 exhibits comparable solubility profiles to the Lys-modified Cp40 derivatives [20], it has distinctly shorter ocular residence than Cp40-KK or Cp40-KKK (unpublished

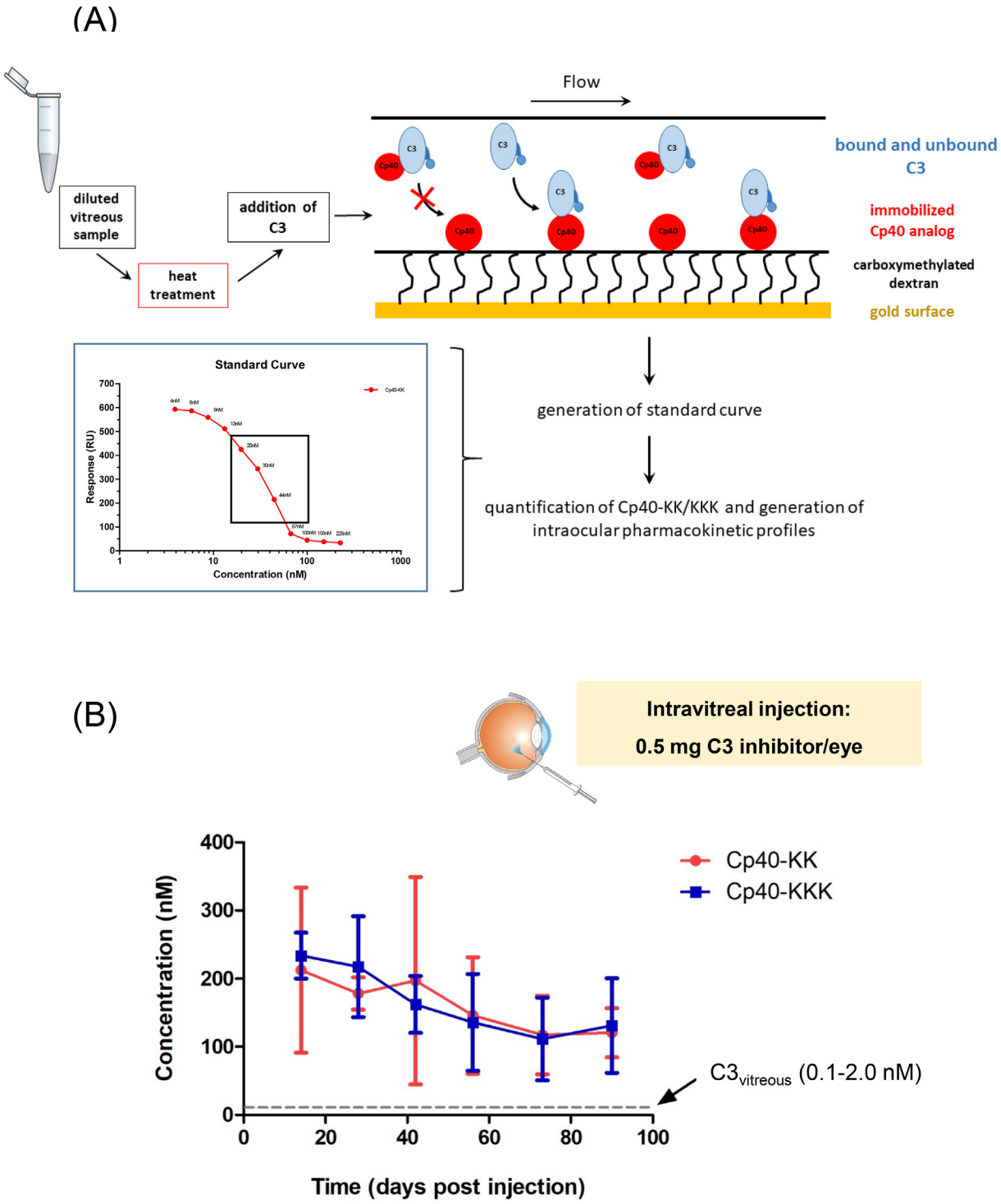

Fig. 1. (*Panel A*): *Development of a sensitive SPR-based platform for quantification of Cp40-based analogs in NHP vitreous samples*. This scheme illustrates the basic steps in the implementation of a highly sensitive detection platform that can quantify trace amounts of Cp40-based analogs in various clinical samples including plasma or vitreous humor. The detection platform exploits the rapid turnaround time, robustness and dynamic range of analyte detection offered by surface plasmon resonance (SPR)-based interaction analysis. To monitor the intraocular PK profile of Cp40 analogs, vitreous samples are collected at predetermined time points and subjected to heat dissociation to release Cp40 analogs from their tight complexes with C3. These vitreous-derived Cp40 analogs are allowed to react with a standard source of C3 (such as pooled human plasma or purified human C3) and thereby compete with biosensor-immobilized Cp40 analogs for binding to free C3. An SPR signal decrease will reflect less binding of free C3 to the sensor surface and is directly proportionate to the amount of Cp40 analog present in the test sample (i.e., vitreous). The SPR-based detection method is described in greater detail in the text.
(*Panel B*): *Pharmacokinetic profile of compstatin-based analogs Cp40-KK and Cp40-KKK in the vitreous of non-human primates*. The intravitreal concentration of the lysine-modified Cp40 analogs, Cp40-KK and Cp40-KKK was determined by SPR-based quantification at various time points (i.e., 14, 28, 42, 56, 73, 90 days) following a single IVT injection of 500 μg of inhibitor in cynomolgus monkeys. A total of three eyes were used per treatment (inhibitor) and each data point in the curve represents the mean value ± SD from $N = 3$ animals per group. Each curve represents successive PK measurements (intraocular inhibitor levels) from the same animal, averaged for a group of three treated eyes per inhibitor.

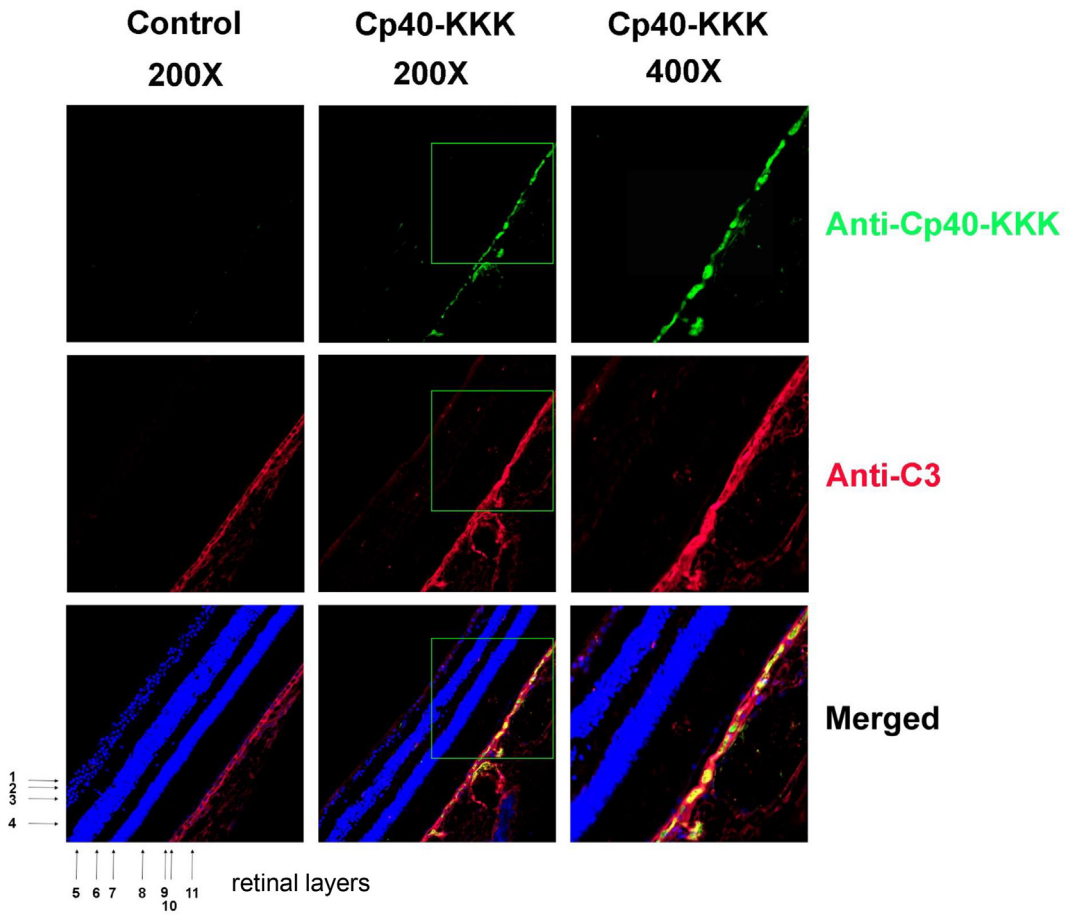

Fig. 2. Retinal tissue distribution of Cp40-KKK and its co-localization with C3 in NHPs.
Non-human primates (NHPs) were treated with one dose of 500 μg of Cp40-KKK peptide via intravitreal Injection. One month later following the injection, the NHPs were euthanized. Both right and left eyes of NHPs were collected, fixed in 4% paraformaldehyde solution in PBS and held at 4 °C until dissection. The eyecup was carefully dissected into two halves; the eyecup half with the optic nerve head was cryopreserved overnight in 30% sucrose (prepared in PBS) at 4 °C. The following day, the sample was embedded in OCT, frozen in isopropanol (precooled in dry ice), and the tissue was sectioned at 12-μm thick sections and stored at -80 °C. The sections were then stained with both goat anti-human complement C3 (1:200 dilution) and anti-Cp40 antibody (final concentration of 2 μg/ml) as described in 'Materials and Methods'. The Cp40-KKK staining signal was amplified by using the Alexa Fluor™ 555 Tyramide SuperBoost kit (Thermo Fischer, B40923). The images were taken using a Nikon Eclipse Ti2 Confocal Microscope. Note: Control: Retina tissue was from untreated NHPs. The middle (Cp40-KKK, 200×) and right panel (Cp40-KKK, 400×) of photomicrographs display retinal tissue sections from NHPs being treated with one dose of 500 μg of Cp40-KKK peptide at x200 and x400 magnification, respectively; the green-colored squares in the middle panel denote the magnified region of the retina. Red color: represents the C3 staining; Green color: represents the Cp40-KKK staining. Blue color: represents DAPI staining of nuclei. Yellow color: represents the co-localization of both C3 and Cp40-KKK. The retinal tissue consists of a total of 11 layers: 1. Internal limiting membrane; 2. Nerve fiber layer; 3. Ganglion cell layer; 4. Inner plexiform layer; 5. Inner nuclear layer; 6. Outer plexiform layer; 7. Outer nuclear layer; 8. Layer of rods and cones; 9. Retinal pigment epithelium; 10. Bruch's membrane; 11. Choroid.

observations). Of note, while PEGylation is a method of choice for extending the plasma half-life of peptide therapeutics, as exemplified by the addition of a 40 kDa PEG moiety to Cp40, it may entail an undesirable trade-off involving a significant decrease of binding affinity to C3 by at least two orders of magnitude, as measured by SPR-based kinetic analysis [31].

Interestingly, the amount of Cp40-KKK detected in the vitreous of treated cynomolgus monkeys on day 14 represents approximately 0.2% of the initially injected dose, which indicates that the majority of the injected compound is rapidly cleared from the eye and only the compound that is bound to its target remains longer in the tissue. This also suggests that IVT administration with these Cp40-based analogs may achieve the observed residence time at much lower doses. The higher intravitreal concentration of Cp40-KKK, relatively to C3, indicates that the compound may be retained in the vitreous through a yet unidentified mechanism. The possibility that a fraction of the compound, already distributed within various ocular tissues, may be released back into the vitreous (e.g. 'depot' effect) cannot be ruled out. While plausible, all these processes remain speculative and warrant further investigation.

Considering the prolonged intraocular residence of the lysine-modified Cp40 analogs (Cp40-KK and Cp40-KKK) and potential drug accumulation effects elicited through repeated injections, it is conceivable that these novel C3 therapeutics could be suitable candidates for reducing the frequency of drug dosing in retinal patients down to an interval of 5–6 months between each treatment. This would constitute a major stride towards a more patient-compliant intraocular peptide therapeutic. These observations further point to a significant improvement in drug dosing with regard to the ongoing phase III trials of APL-2/Pegcetacoplan, in which GA patients are being dosed with this PEGylated compstatin derivative at one- or two-month intervals [7,17]. Another issue that is worth considering in these translational efforts is the risk associated with the chronic exposure of the retinal tissue to ocular therapeutics that incorporate PEG moieties for achieving reduced clearance (e.g. as in the case of APL-2 or Zimura). A high PEG burden or potential PEG accumulation in the retina, as a result of repeated IVT injections, could drive off-target effects, likely even modulating the CNV conversion rate observed in the GA patients treated

with APL-2 or Zimura [18,32]. Ongoing clinical trials evaluating C3 or C5-targeted therapeutics are expected to reconcile these initial observations with more concrete clinical evidence that will weigh on the potential contribution of these confounding factors to the clinical course of AMD.

Cp40-KKK exhibited markedly prolonged intraocular residence at C3-saturating levels, extending over 90 days after a single intravitreal injection. This C3 therapeutic showed considerable retinal tissue penetrance and target-driven distribution with pronounced co-localization with C3 in the choroid of injected animals. These findings have important implications for the design of C3-targeted ocular therapeutics tailored for chronic intravitreal administration in patients with retinal diseases underpinned by deregulated C3 activity (e.g. AMD). To enable the accurate PK monitoring of compstatin Cp40-based analogs within the ocular compartment we developed a highly sensitive and robust SPR-based bioanalytical method. Using this detection platform, we determined favourable intraocular PK profiles of Cp40-KK and Cp40-KKK in cynomolgus monkeys injected intravitrealy with a single dose of inhibitor.

In summary, we have shown that the fourth-generation compstatin analog Cp40-KKK, displays a highly favourable intraocular PK and tissue distribution profile that support its clinical development as a C3-targeted therapeutic for ophthalmic indications. Encompassing a unique combination of druggable features including its small size, high target binding affinity, enhanced solubility, and prolonged ocular residence, Cp40-KKK constitutes a promising drug candidate for C3 modulation in the ocular compartment. Cp40-KKK is thus expected to allow for development of new C3-targeted therapies that could offer broader and sustained complement control in retinal diseases, as well as enhanced patient compliance through reduced drug dosing frequency.

Declaration of Competing Interest

J.D. Lambris is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for therapeutic purposes. J.D. Lambris and D. Ricklin are inventors of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. J.D. Lambris is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals (i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan). G. Hajishengallis has a patent that describes the use of complement inhibitors for therapeutic purposes in periodontitis. The other authors declare no competing interest.

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