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Article

A Controlled Release System for Long-Acting Intravitreal Delivery of Small Molecules

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Purpose: The short half lives of small molecules in the vitreous requires frequent repeated intravitreal injections that are impractical for treatment of chronic eye diseases. We sought to develop a method for increasing the intravitreal half-life of small-molecule drugs.

Methods: We adapted a technology for controlled release of drugs from macromolecular carriers for use as a long-acting intravitreal delivery system for small molecules. As a prototype, a small molecule complement factor D inhibitor with an intravitreal half-life of 7 hours was covalently attached to a 4-arm PEG_{40kDa} by a self-cleaving β-eliminative linker with a cleavage half-life of approximately 1 week.

Results: After intravitreal injection in rabbits, the drug was slowly released in the vitreous, and equilibrated with the retina and choroid. The intravitreal half-life of the intact PEG-drug conjugate in the rabbit was 7 days, and that of the released drug was 3.6 days. We simulated the anticipated pharmacokinetics of the delivery system in human vitreous, and estimated that the half-life of a 4-arm PEG_{40kDa} conjugate would be approximately 2 weeks, and that of the released drug would be approximately 5 days.

Conclusions: We posit that a linker with a cleavage half life of 2 weeks would confer a half life of approximately 7 days to a released small molecule drug in humans, comparable to the half life of approved intravitreal injected macromolecular drugs.

Translational Relevance: With this technology, a potent small molecule with an appropriate therapeutic window should be administrable by intravitreal injections in the human at once-monthly intervals.

Introduction

Intravitreal (IVT) injection has become an important drug delivery modality for many diseases of the eye. IVT injection minimizes systemic exposure and allows intraocular drug exposures not otherwise achievable. However, because IVT therapy is an invasive procedure that often requires long-term serial injections, IVT drugs require long IVT half lives ($t_{1/2}$) and dosing intervals.

Macromolecules show low clearance in the vitreous that is related to their molecule diffusivity through the chamber[1]; indeed, it has been shown that the hydrodynamic radius (R_H) of a macromolecular drug is directly related to its half-life in the rabbit vitreous. Currently used IVT drugs include large proteins—a Fab (ranibizumab), mAb (bevacizumab), and a Fc-receptor conjugate (aflibercept)—having IVT half lives of 7 to 10 days in a human (Table 1). PEGylation of aptamers and smaller proteins has also been used to increase the hydrodynamic radius of drugs and IVT half-lives. Successful examples include a PEGylated aptamer (pegaptanib) and a small protein DARPin (abicipar pegol); interestingly, despite its smaller size, the PEGyated protein abicipar has a longer vitreous half-life than larger macromolecular proteins. In short, the current drugs for IVT

Table 1. Properties of IVT Drugs in The Rabbit and Human Vitreous

Drug	MW, kDa	R_H[1]	Rabbit $t_{1/2}$, d[1–8]	Human $t_{1/2}$, d[1–8]	Human t_{DI}[a], wk
Ranibizumab[2,3]	48	2.8	2.5	9	4
Aflibercept[2,4]	115	5.2	3.6	7	4, 8[b]
Bevacizumab[2,5,6]	148	5.2	7.0	10	4
Abicipar pegol[7]	34	NA	6	13	4, 8[c]
Pegaptanib[8]	49	7.3	3.8	10	6

NA, not available.
[a] t_{DI}, dosing interval.
[b] Aflibercept is dosed q4w for 3 doses, then q8w (Eylea label).
[c] In Phase II trials abicipar pegol was dosed q4w for 3 doses, then q8w or q12w.

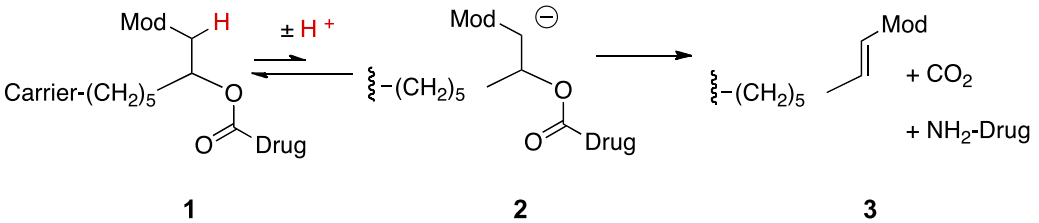
injection have IVT half-lives clustered between approximately 7 and 13 days in a human. The 4- to 8-week dosing interval commonly used for IVT-administered macromolecular drugs requires that they transit multiple half-lives and have high peak-to-trough ratios each dosing cycle; however, this is tolerable because these drugs have wide therapeutic windows, and few off-target effects in the eye.

In contrast to macromolecules, small molecules and peptides have high clearance in the vitreous. With IVT half lives of only approximately 1 to 24 hours, unless formulated as sustained-release implants, they require frequent repeated IVT injections that are impractical for treatment of chronic eye diseases. Because there are so many potential therapeutic targets in the eye,[9,10] there is a large unmet need for a simple, effective way to increase the residence time of small molecules in the vitreous.

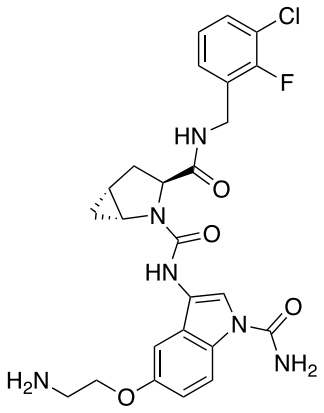
A possible approach to extend the IVT half life of such drugs is to attach them to a soluble macromolecular carrier, such as PEG, to effectively increase their hydrodynamic radius, and thus half life. PEG provides the additional benefit of solubilizing hydrophobic small-molecule drugs. However, whereas attachment of PEG to larger multifunctional drugs, such as aptamers or small proteins, can be achieved at nonessential sites of the drug, attachment of PEG to a small molecule having only a few functional groups is likely to impair activity. One approach to circumvent this problem is to convert the small molecule drug to a macromolecular prodrug that will retain a long IVT half-life because of large hydrodynamic radius, and slowly release the native, pharmacologically active small drug over a long duration.

We have developed a general prodrug approach for half-life extension of therapeutics in which a drug is covalently tethered to a long-lived carrier, such as PEG, by a linker that slowly self-cleaves to release the native drug.[11] Here, a macromolecular carrier is attached to a linker that is attached to an amine group of a drug or a prodrug via a carbamate group (**1**; **Scheme 1**); the β-carbon has an acidic carbon–hydrogen bond (C–H) and also contains an electron-withdrawing "modulator" (Mod) that controls the pK_a of that C–H. Upon hydroxide ion-catalyzed proton removal, a rapid β-elimination occurs to cleave the linker-carbamate bond and release the free alkene products, **2**. The rate of drug release is proportional to the acidity of the proton, and that is controlled by the chemical nature of the modulator; thus, the modulator chemically controls the drug release rate in a predictable manner.

We have previously shown that approximately 40 kDa PEGylated fluorescent conjugates show an IVT half life of approximately 7 days in the green monkey without apparent toxicity.[12] In the present work we describe the preparation and characterization of a releasable PEGylated prodrug of DS29740219, a



Scheme 1.



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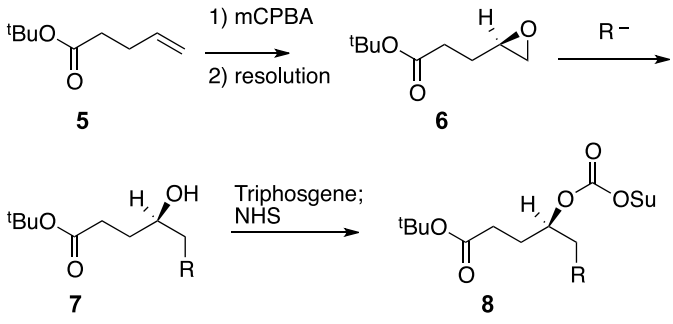
potent small molecule inhibitor of complement factor D (CFD) that has potential as a therapeutic for dry age-related macular degeneration (AMD).[13,14] We also report the intravitreal pharmacokinetics of the macromolecular prodrug, which indicates that the released drug can be kept above its IC_{50} for up to approximately 1 month. The results indicate that PEGylated prodrugs may provide a practical, general technology platform for discovery and delivery of long-acting small molecule drugs for IVT injection.

Materials and Methods

The source of specialized materials is provided along with their use in Supplementary Materials. Detailed synthetic, conjugation, and analytic procedures are described. In vitro kinetic procedures are provided, as are in vivo pharmacokinetic methods and analyses.



Scheme 2.

Results

Chemistry

Synthesis of DS29740219 (4)

The aminoethyl analog **4** was prepared by catalytic reduction (Pd/C) of the reported corresponding cyanomethyl precursor.[15] Compound **4** showed a half-maximum inhibitory concentration (IC_{50}) of 53 nM for in vitro inhibition of CFD, and 95 nM in an alternate pathway-mediated hemolysis assay.

Synthesis of PEG-4 Conjugates

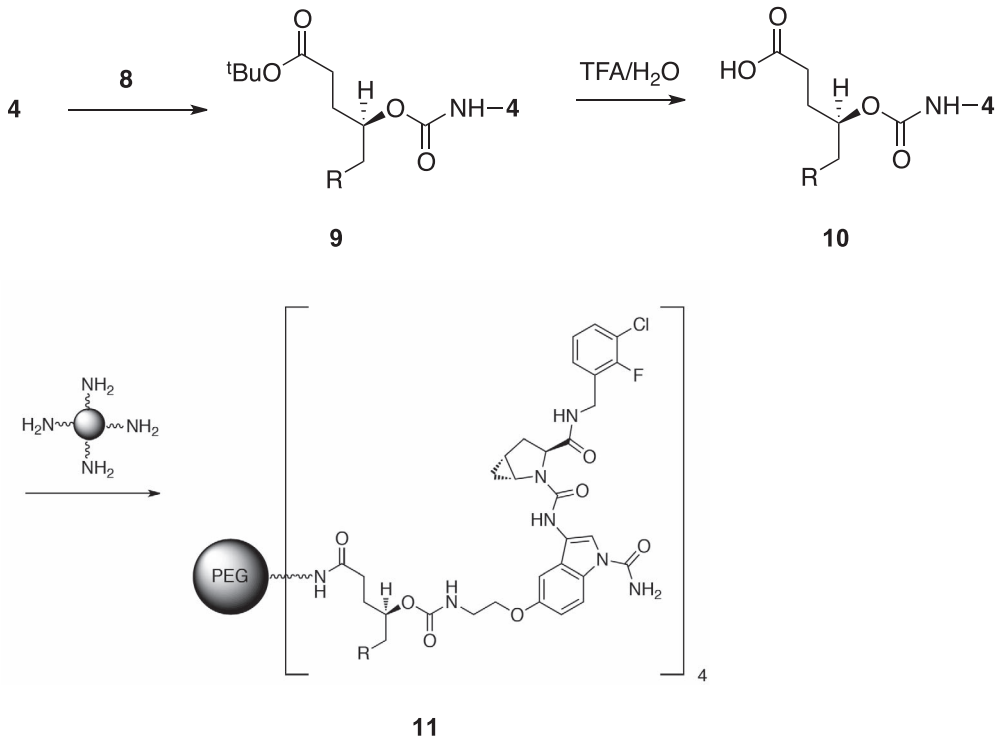
As depicted in Scheme 2, treatment of commercially available 4-pentenoic acid with isobutylene/H_2SO_4 gave ^{t}butyl 4-pentenoate **5**, which was epoxidized with mCPBA. The epoxide was readily resolved using Jacobsen's kinetic resolution.[16] The resolved epoxide **6** was opened with a nucleophile ($MeSO_2Na$ for $MeSO_2$ and NaCN for CN) to introduce the linker modulator group. The resulting linker-alcohols **7A,B** were activated in a two-step process, first converting them to the chloroformates (triphosgene, pyridine) and then to the hydroxysuccinimidyl carbonates **8A,B** (HOSu, pyridine).

Reaction of **4** with **8A,B** to provide **9A,B** was rapid and quantitative, and removal of the ester group cleanly provided the linker-drugs **10A,B** ready for conjugation with PEG-amine (Scheme 3). Conjugation was most conveniently performed by in situ activation of the linker-drug using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU), and the conjugates **11A,B** were isolated by dialysis followed by precipitation to remove small molecule impurities. In contrast to the poor water solubility of **4**, the PEG-conjugates **11A,B** were soluble in water at more than 100 mg/mL.

A corresponding nonreleasable conjugate **12** was prepared as a control by reacting **4** with PEG_{40kDa} tetra(succinimidyl carboxymethyl ester) (Scheme 4). As with **11A,B**, the PEG-conjugate **12** was soluble in water at more than 100 mg/mL.

In Vitro Release Kinetics

The conjugates were examined for release of **4** under accelerated in vitro conditions at pH 8.4, 37°C, using high-performance liquid chromatography (HPLC) analysis (Fig. 1). The data were fit to first-order release profiles, giving observed half-life values of 17.5 (**11A**) and 90 hours (**11B**) that, assuming hydroxide-catalyzed cleavage,[11] extrapolate to 175 and 900 hours, respectively, at pH 7.4. Because the



Scheme 3.

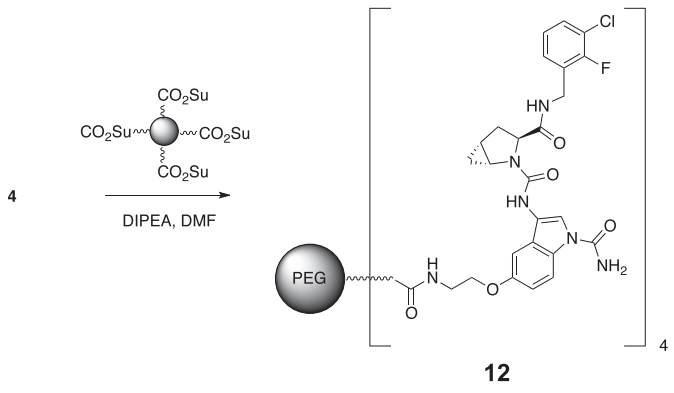
cleavage rate of **11B** was much slower than the expected IVT rate of elimination of PEG_{40kDa} it was deemed unsuitable for IVT use. With the stable conjugate **12**, no release of **4** was observed for at least 200 hours at pH 8.4.

IVT Pharmacokinetics

IVT Models and Pharmacokinetic Data in the Rabbit

We have previously reported kinetic analyses of a one-compartment model for β-eliminative release of drugs from soluble macromolecular carriers.[11] As in Scheme 5, the drug is released from the conjugate in a first-order process with rate constant k_1, the drug is cleared with k_2, and the conjugate is cleared with rate constant k_3. If $k_2 \gg (k_1 + k_3)$, at longer times the slopes of plots of ln[PEG-Drug] and ln[Drug] versus time both approach $k_1 + k_3$. If the clearance rate (k_3) of the conjugate is known, it can be subtracted from the slope of the ln[PEG-Drug] versus time plot ($k_1 + k_3$), to obtain the in vivo linker cleavage rate constant, k_1.

To estimate k_2, parent compound 5 ug of **4**/eye was administered by IVT injection of New Zealand White rabbits and concentrations in the vitreous were



Scheme 4.

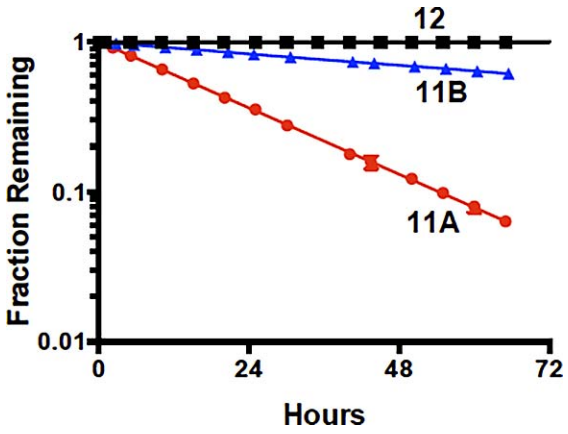
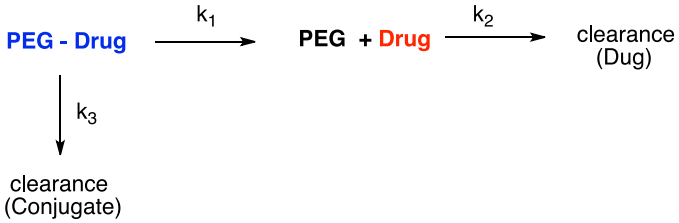


Figure 1. In vitro release kinetics of **11A** (R = $MeSO_2$, *red circle*), **11B** (R = CN, *blue triangle*) and **12** (*black square*) at pH 8.4, 37°C. Points are averages of duplicate determinations and data were fit to first-order release profiles. *Error bars* show standard deviation (SD).

measured over time using HPLC–tandem mass spectrometry. The C versus t plot shows a brief distribution phase, followed by a first-order elimination of **4** from the vitreous (Fig. 2). Analysis of the C versus t plot using a 2-compartment model (*SI*) indicated a terminal $k_{e,IVT} = 0.31\ hr^{-1}$ and $V_d = 0.57$ mL, with intercompartmental IVT transfer rates of $k_{12,IVTout} = 0.20\ hr^{-1}$ and $k_{21,IVTin} = 0.17\ hr^{-1}$; one-compartment analysis, with weighting $1/Y^2$, showed an IVT terminal half life of 6.9 hours.

To estimate the elimination rate, k_3 of the releasable PEG-**4** conjugate, we used the corresponding stable conjugate **12** as a surrogate. Conjugate **12** (465 nmol of **4**) were administered by IVT injection to each eye of the subject rabbits, and the IVT levels were measured over 28 days (Fig. 3A). Here, the IVT half life was 167 hours in the rabbit, which exceeds the 90-hour IVT half life of the PEGylated aptamer pegatanib and the 144-hour IVT half life of the long-acting PEGylated DARPin, abicipar pegol (Table 1). Very low levels (<0.01% of **12**) of **4** were also detected but did not impact our conclusions so we did not investigate its origin.

The IVT C versus t plot of releasable conjugate **11A** (465 nmol **4**/eye) as well as free **4** released from **11A** over 28 days is shown in Figure 3A. We analyzed the data as a one-compartmental model (below) as well as a multicompartment model (*SI*) to accommodate the initial brief distribution phase. Both models provide a similar terminal IVT elimination rate of $k_{\beta,IVT} = 0.19\ d^{-1}$ ($t_{1/2} = 86$ hours or 3.6 days). Because the IVT elimination rate, $k_{IVT,}$ of **11A** represents the sum of the prodrug elimination and drug release



Scheme 5.

rates,[11] $k_{IVT} = k_1 + k_3$, using k_3 determined for **12** we calculate an IVT linker cleavage rate, k_1, of 0.094 $days^{-1}$. The 177-hour half life for release of **4** from the releasable conjugate **11A** in the vitreous is in excellent agreement with the in vitro cleavage half life of 175 hours described above. As expected, the C versus t plot of released **4** in the vitreous parallels that of **11A** with a similar half life of 2.9 days (69 hours).

The efficiency of drug utilization from a prodrug, such as **11A**, can be described by the partition ratio (PR), $k_1/(k_1 + k_3)$,[17] which represents the fraction of the prodrug that releases **4** during its IVT residence. In the present case, **11A** shows a high efficiency of 0.74.

We also analyzed the **4** released from **11A** in the retina and choroid, because these are the target ocular structures of the CFD inhibitor; the C versus t plots are shown in Figure 3B, along with the IVT C versus t data of released **4** reproduced from Figure 3A. Higher levels of **4** were observed in the retina compared with the vitreous, but the terminal elimination rates were similar. In the choroid, lower levels of **4** formed from **11A** were present compared with the vitreous or retina; however, the terminal elimination rate of **4** was almost 2-fold slower than from the vitreous or retina.

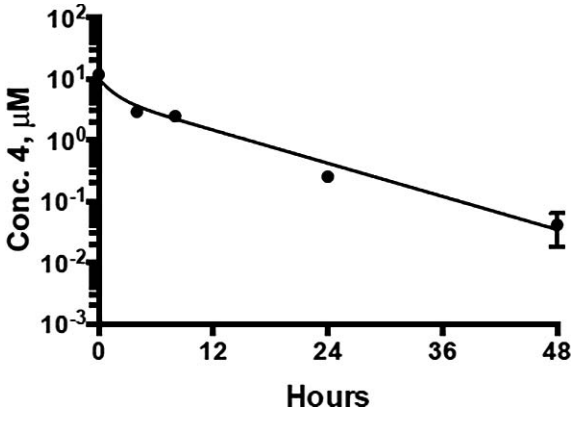


Figure 2. IVT C versus t plot of **4**, showing the terminal half life of 6.9 hours for **4**. Points are averages of two determinations and *vertical bars* show the range of concentrations measured.

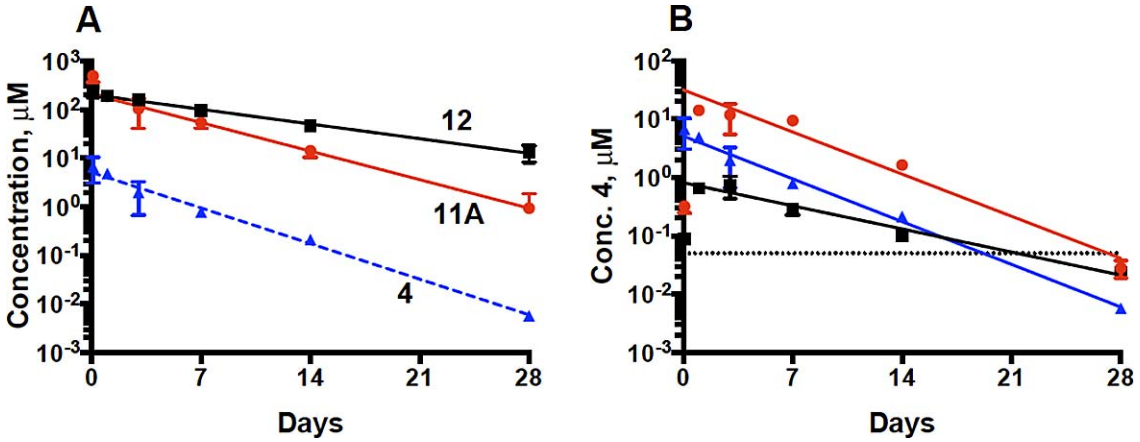


Figure 3. C versus t plots showing terminal half-lives of PEGylated and free **4** in the vitreous. (A) Stable **12** (*black square*), releasable **11A** (*red circle*), and free **4** (*blue triangle*) in vitreous, and (B) free **4** in the vitreous (*blue triangle*), retina (*red circle*), and choroid (*black square*); equilibration phases not shown. Points are averages of two determinations and *vertical bars* show the range of concentrations measured. The *dotted line* represents the IC_{50} of CFD inhibition by **4**.

Simulations in the Human Eye

With several reasonable assumptions, we can simulate how a releasable PEG_{40kDa}-drug conjugate studied in the rabbit would behave in the human eye. As described above, the half life of a drug released from a PEG-drug conjugate by a β-eliminative linker ($k_{el} = k_1 + k_3$) is driven by the rate of the linker cleavage (k_1) and the elimination of the PEG_{40kDa} conjugate (k_3). Here, k_1 is species independent and can be controlled over a wide range by the linker used.[11] However, the rate of elimination of the carrier, k_3, is species specific and with slow-cleaving linkers where $k_3 > k_1$ represents a theoretic upper limit for the IVT half life of the released drug. Shatz et al.[1] have recently shown that the IVT half life of macromolecules in the rabbit show a linear correlation with R_H with a slope of 0.65 days/nm R_H. In the rabbit, the IVT half life of the stable PEG_{40kDa} conjugate **12** is 7 days, which agrees well with the 6 days predicted for a macromolecule with R_H 9.3 nm.[18] In a human, the half-life values of all Food and Drug Administration approved IVT macromolecules is 1.8- to 2.8-fold (average 2.2) longer than in the rabbit (Table 2). Hence, the IVT half life of an intact PEG_{40kDa} conjugate (e.g., **12**) in a human should be approximately 2 weeks, and from $k_{IVT} = k_1 + k_3$ the $t_{1/2,IVT}$ in the human of **11** and the **4** released from **11** should be approximately 112 hours. If we were to use a linker with a cleavage rate of 2 weeks, the $t_{1/2,IVT}$ of **4** released from a PEG_{40kDa}-conjugate in the human vitreous should be approximately 7 days.

Discussion

The primary objective of this work was to determine the IVT half-life extension that could be achieved for a small molecule released from a 4-arm PEG_{40kDa}-drug conjugate after IVT injection.

Four-arm PEG_{40kDa} was chosen as the nanocarrier for the following reasons: (1) PEG conjugation increases the water solubility of hydrophobic small-molecules, (2) a four-arm PEG_{40kDa} polymer has a high R_H of 9.3 and provides long IVT half-life

Table 2. Pharmacokinetics of **4**, **11A** and **4** Released From **11A** in the Rabbit Eye

Compound	$t_{1/2}$, hr	$t_{1/2}$, d	CL, mL/d[a]	V_{dss}, mL[a]	C_{max}, uM
4					
Vitreous	6.9	0.29	3.5	0.91	
12					
Vitreous	170	7.0	0.22	2.1	
11A					
Vitreous	86	3.6	0.36	1.6	210[b]
4 from **11A**					
Vitreous	69	2.9	NA	NA	6.7
4 from **11A**					
Retina	60	2.5	NA	NA	14
4 from **11A**					
choroid	140	5.9	NA	NA	0.70

NA, not applicable.
[a] Calculated from noncompartmental analysis.
[b] Calculated as C_0 for **11A**.

extension, (3) four equivalents drug can be attached per equivalent of carrier to minimize the volume of IVT injection, and (4) high molecular weight PEG shows little or no IVT toxicities.

Compound **4** was prepared as a prototype small-molecule inhibitor of CFD for potential treatment of geographic atrophy resulting from AMD. An IC_{50} of approximately 50 nM was determined for **4** in an in vitro inhibition assay of human CFD and approximately 90 nM in an alternative pathway-mediated hemolysis of rabbit erythrocytes. The molecule also possesses a primary amine for convenient chemical attachment to β-eliminative linkers by a carbamate group.

The CFD inhibitor **4** was appended to the ends of four-arm PEG_{40KDA} through a releasable β-eliminative linker with a $MeSO_2$ modulator to give **11A,** or through a stable linker to give **12**. In vitro, the half-life for cleavage of the linker in **11A** was 175 hours at pH 7.5, 37°C; in contrast, **12**, which lacks a β-eliminative linker, was inert. When the stable PEG-conjugate **12** was injected IVT in the rabbit and the vitreous concentration measured over time, we observed a half life of 7 days, close to the half life of 6 days predicted from its R_H.[1] With the cleavable conjugate **11A**, the linker cleavage half life was 177 hours in the vitreous, and the IVT half life was 3.6 days; expectedly, the released **4** from **11A** showed a similar apparent IVT half life of approximately 3 days.

Within approximately 1 day after IVT injection, the inhibitor **4** released from **11A** reached equilibrium in the retina and choroid, the presumed locations of the target CFD. The C_{max} of **4** in the retina was approximately 2-fold higher than in the vitreous, and approximately 20-fold higher than the choroid. However, whereas the elimination rate of **4** from the retina was similar to that in the vitreous, elimination from the choroid was approximately 2-fold slower.

The IVT half-life extension achieved by β-eliminative release of **4** from **11A** compared with direct injection of **4** is approximately 10-fold in the rabbit (i.e., the half life is 7 hours for IVT-injected **4** versus 70 hours for **4** released from the PEG-drug conjugate **11A**). This magnitude of half-life extension should translate to other molecules attached to PEG_{40kDa} by the same β-eliminative linker because the half life of released drugs is driven by the rate of the linker cleavage and the elimination of the PEG_{40kDa} conjugate from the IVT compartment.

Available data indicates that the IVT half life of macromolecules in humans are approximately 2-fold longer than the corresponding IVT half life in rabbits. Thus, we could estimate that the IVT half life of a four-arm PEG_{40kDa} in a human would be approximately 2 weeks, and comparable to that of abicipar pegol, the long acting PEGylated DARPin. For the linker with cleavage half life of 177 hours used here, we calculate from $k_\beta = k_1 + k_3$ that the IVT half life in a human of the released drug should be approximately 4.8 days. However, if a β-eliminative linker with a cleavage half life of 14 days were used, the IVT half life in humans should be approximately 1 week, comparable to that of most macromolecular IVT drugs. Thus, providing the therapeutic window of a drug is sufficiently wide to allow four half lives with $C_{max}/C_{min} \geq 8$, the drug delivery system described here should allow a once monthly or longer dosing interval of a small molecule.

In summary, IVT injection of a PEG_{40kDa} nanocarrier attached to a small molecule drug via a releasable β-eliminative linker could maintain the released drug at therapeutic levels in the vitreous for periods comparable to approved IVT macromolecular drugs (i.e., 1 month or longer). The technology described here could serve as a screening platform for identifying potent small molecules that may have therapeutic utility in the eye. This could provide a large benefit of determining pharmacodynamic effects of a long-acting small molecule before investing efforts in developing a final delivery system. Further, once a drug with appropriate efficacy and therapeutic window is found, the releasable PEG_{40kDa}-drug platform could serve as a delivery system in the therapeutic formulation. The largest limitations of the technology might be the capacity of the carriers, or the IVT therapeutic window of the released drug. Finally, even longer IVT half-life extension of small molecules might be achieved using β-eliminative linkers tethered to carriers with longer IVT half lives. Potential candidates for such carriers might include hyaluronic acid with a $t_{1/2,IVT}$ approximately 30 days,[19] the recently reported long-lived L-Arg peptide-conjugated nanocarriers,[20] or previously described Tetra-PEG hydrogel microspheres[21] attached to drugs via β-eliminative linkers.

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*NM and GWA contributed equally to this work.

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