THE INTRAVITREAL PHARMACOKINETICS OF FLUOROPHORES CONJUGATED TO PEGs BY NON-CLEAVABLE AND SELF-CLEAVING LINKERS IN NONHUMAN PRIMATES

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Objectives

To establish a delivery platform that achieves tunable drug release by employing a novel "self-cleaving" linker chemistry to conjugate drugs to carrier polymers by:

1. Determining the dependence of polyethylene glycol (PEG) pharmacokinetics on the size and branch number in the nonhuman primate eye by non-invasive fluorophotometry (FP).
2. Evaluating the pharmacokinetics of intravitreally (IVT) delivered PEG attached by self-cleaving linker to either Tokyo green or a FRET pair (fluorescein/4-([4-(Dimethylamino)phenyl]azo)benzoate (fluorescein/DABCYL)) to characterize the release properties of the PEG fluorescein conjugates, and define posterior-anterior fluorescein concentrations and distribution following intraocular administration.

Background

Drug conjugation to macromolecules that are well-tolerated and have long residence times via nonenzymatic β-elimination reaction with preprogrammed, highly tunable cleavage rates present a strategy to improve pharmacokinetics of existing and candidate ocular therapies[1,2]. Utilizing a fluorescein-linked conjugate molecule, fluorophore release and distribution can be quantified non-invasively with fluorophotometry (FP) to model ocular drug release behavior.

Methods

1. Ophthalmic examinations including fluorophotometry were performed on 15 healthy adult green monkeys (*Chlorocebus sabaeus*). All examination procedures were performed following sedation by intramuscular administration of ketamine (8 mg/kg) and xylazine (1.6 mg/kg). Any animals displaying evidence of ocular pathology or anomalous autofluorescence were excluded. Initially 3 animals received IVT injection of 80k linear, non-cleavable PEG fluorescein conjugate (100 μL of 600 μM fluorescein conjugate) OD and unconjugated fluorescein (100 μL of 600 μM fluorescein in 0.9% saline) OS to characterize the PK of both the linear PEG conjugated and unconjugated fluorescein. Next, 3 groups of 4 animals received either a) 20K linear PEG fluorescein conjugate OD and 40k 2-branched PEG fluorescein conjugate OS, b) 40k linear, PEG fluorescein conjugate OD and 40k 3-branched PEG fluorescein conjugate OS, or c) 80k linear PEG fluorescein conjugate OD and 40k 4-branched PEG fluorescein conjugate OS by IVT injection. All PEGs in this series were again bound to fluorescein by non-cleavable linkers and administered at a dose of 50 μL of a 600 μM formulation. IVT injections were performed with a 0.3 cc insulin syringe with a 31-guage 0.5 inch needle and placed 3mm posterior to the limbus in the inferior temporal quadrant. Slit lamp exams and FP were performed at set time points post IVT dose.
2. Ophthalmic examinations including FP were performed on 4 healthy adult green monkeys as above. Each animal received IVT injection in each eye consisting of 50 μL of 300 μM PEG Tokyo green fluorescein conjugate designed to fluoresce when released from the PEG, or 50 μL of 600 μM PEG FRET pair conjugate designed to fluoresce with the release of a quencher attached by a cleavable linker while fluorescein remains bound to the PEG.

Fluorophotometry was performed on a FM-2 Fluorotron Master (OcuMetrics, Mountain View, CA) under ketamine and xylazine sedation. Scans were performed every 0.25 mm from the retina to the cornea along the visual axis. The area under the curve (AUC) of the posterior peak and aqueous peak were calculated using the trapezoidal rule and expressed in units of fluorescence times the anterior-posterior distance.

Results

PEG pharmacokinetics not strongly dependent on size and branch number in the 40 kilodalton range

High molecular weight fluorescein conjugated PEGs of different sizes and branching pattern remained in the vitreous for prolonged periods with no signs of adverse effects.

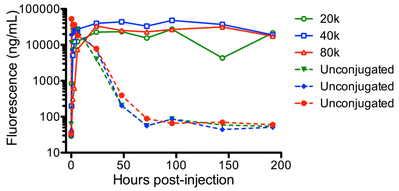

Figure 1: Vitreous chamber fluorescence of 20K, 40K, and 80K linear PEG fluorescein conjugates versus unconjugated fluorescein. The PEG conjugated fluorescein exhibited prolonged retention in the eye (n = 3 per group) as evidenced by the sustained fluorescein signal compared with unconjugated fluorescein.

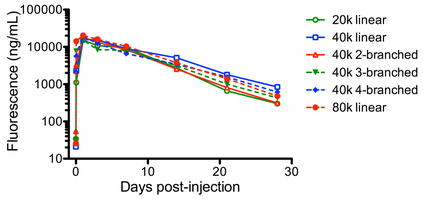

Figure 2: Vitreous chamber fluorescence area under the curve (AUC) of 20K, 40K, and 80K linear and 40K 2, 3, & 4 branched PEG fluorescein conjugates. Intraocular PEG conjugate retention is not highly dependent on PEG size or branching pattern among the configurations explored, supporting the use of a four branched PEG ("tetra PEG") that would accommodate more drug conjugation sites per PEG molecule.

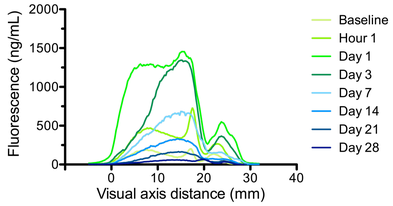

Figure 3: Ocular fluorescence signal of the 40K 4-branched PEG along the visual axis over successive FP measures from baseline to day 28. The distribution and time course of fluorescent signal (n = 4) of the candidate tetra PEG used to conjugate by cleavable linkers to fluorophore drug surrogates and drugs.

Modeling

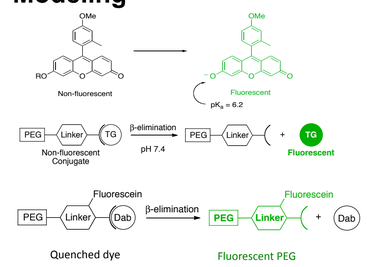

Figure 4: Schematic of Tokyo green fluorescence activation and releasable Tokyo green PEG conjugate

Figure 5: Schematic of quenched and unquenched releasable FRET pair PEG conjugate

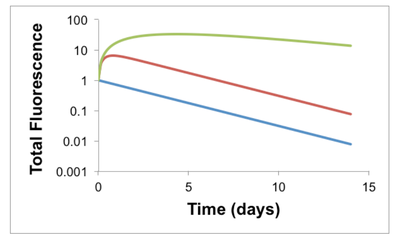

Figure 6: Simulated vitreous humor fluorescence for IVT injected stable PEG fluorescein conjugate (blue) cleavable PEG Tokyo green conjugate in which the fluorophore is released (red) and PEG FRET pair conjugate in which the fluorophore is retained and quencher released (green).

PK of fluorophore drug surrogates tuned by self-cleaving linker design

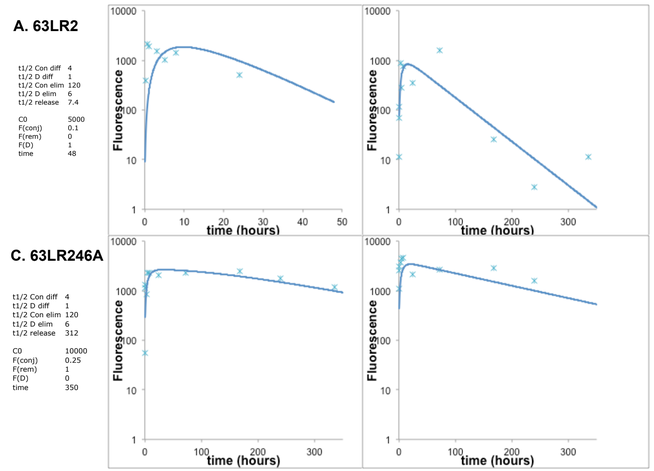

Figure 7: Ocular fluorescence signal of Tokyo green and FRET pair conjugated by self-cleaving linker to 40K tetra PEG. Tokyo Green conjugated by two different linkers (**A**) and (**B**) demonstrated a rapid increase in fluorescence upon IVT injection that decayed at a rate dependent upon the release rate of the conjugate and the clearance rate of the conjugate from the eye. Data were in agreement with model predictions using *in vivo* diffusion and elimination rates derived from fluorescein and stable PEG-fluorescein conjugates together with release rates determined from *in vitro* experiments. The FRET pair with quencher conjugated by two different linkers (**C**) and (**D**) showed a fluorescence signal that decayed at a rate dominated by the clearance rate of the conjugate and again were in agreement with model predictions.

Conclusions

1. PEGs behave as appropriate supports to extend the ocular pharmacokinetics of conjugated drug surrogates or drugs for up to two to three weeks.
2. Conjugation of molecules to a carrier such as PEGs by "self-cleaving" linkers allows further control of the release of the conjugated molecule.
3. The use of fluorophores as drug surrogates allows the characterization of self-cleaving PEG conjugate pharmacokinetics by fluorophotometry in minimally invasive non-terminal nonhuman primate study designs.
4. The binding of drugs by tunable self-cleaving linker to biodegradable macromolecules such as PEG presents a promising strategy for controlled, sustained ocular drug delivery.
5. Further characterization of the achievable drug and/or drug surrogate release and the ocular tolerance of cleavage products of "self-cleaving" linkers is warranted to support pursuit of clinical application of this controlled release strategy.

References

1. Santi, D., Schneider, E., Reid, R. Robinson, L., Ashley, G., Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates PNAS 2013 109:6211-6.
2. Ashley, G., Henise, J., Reid, R., Santi, D. Hydrogel drug delivery system with predictable and tunable drug release and degradation rates PNAS 2013 110:2318-23.