Title: Inhibition of Pathogenic Angiogenesis in Diabetic Retinopathy with a Hyaluronic Acid-based Multivalent VEGF Antagonist

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Motivation: Vascular endothelial growth factor (VEGF) is one of the most potent activators of new blood vessel growth, i.e. angiogenesis[1,2]. Although angiogenesis is a requirement for proper tissue development and wound healing, it is also the primary mediator for accelerating the detrimental effects of blood vessel formation in neovascular diseases such as diabetic retinopathy (DR). Current treatments options available to patients with diabetic retinopathy include pan-retinal laser photocoagulation, which is prohibitively expensive for many patients, and monthly intravitreal injections of VEGF antagonists such as Bevacizumab (Avastin, Genentech) or Ranibizumab (Lucentis, Genentech). These currently available pharmaceuticals for treating DR available anti-angiogenic therapeutics are limited by short half-lives *in vivo* and rapid clearance rate from the site of administration, resulting in the need for frequent injections to maintain an effective dose within the eye[3,4]. In order to increase the tissue residence time of molecular entities, we have recently developed a multivalent conjugate technology through the use of high molecular weight hyaluronic acid (HyA), a naturally occurring glycosaminoglycan in the extracellular matrix, conjugated with the VEGF antagonist, sFlt. As an inhibitor of VEGF signaling, we have synthesized the first 3 extracellular Ig domains of soluble vascular endothelial growth factor receptor-1 (sFlt) through bacterial cloning and insect cell expression, which is able to actively bind and inhibit VEGF activity. The goal of this work is to create an alternative to currently available pharmaceuticals with increased vitreal residence time specifically targeting VEGF mediated angiogenic progression in DR.

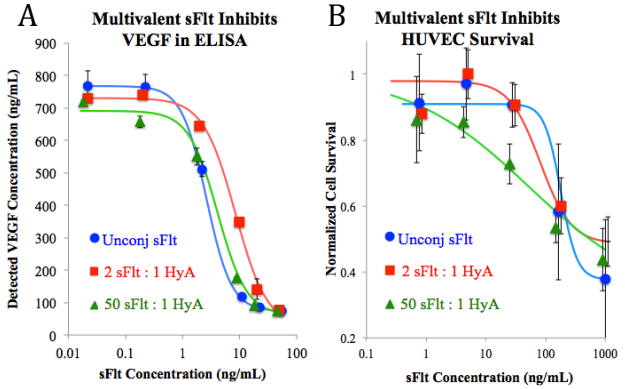

Figure: Unconjugated and conjugated sFlt inhibit VEGF activity in ELISA and HUVEC survival assays. (A) Using a VEGF sandwich-based ELISA assay, we were able to confirm that sFlt is able to inhibit VEGF in both its unconjugated form and its multivalent form, as evidenced by the two conjugation ratios 2:1 and 50:1. (B) Similar results were obtained for VEGF-dependent HUVEC survival. As the concentration of sFlt increased in the media, less VEGF was available to stimulate HUVEC survival.

Methods: sFlt was expressed in the insect cell baculovirus system and purified through a nickel agarose bead chromatography through binding mediated by the 6His tag on sFlt. sFlt was conjugated to HyA through a two-step reaction using carbodiimide chemistry at the carboxylate group and a malemide reaction at the protein C-terminal cysteine which was added to the sFlt to create a site for conjugation uninvolved in protein folding. Feed ratios of sFlt to HyA varied between 1:10 – 1:200. sFlt and multivalent sFlt (mvsFlt) were assayed for inhibition of VEGF using ELISA and *in vitro* HUVEC survival assays.

Summary of Results: Insect cell-expressed sFlt in its unconjugated form was able to inhibit VEGF in both an ELISA based assay and a HUVEC *in vitro* assay. In the figure depicting the ELISA results, as the sFlt concentration increases, the available VEGF for binding to the capture antibodies decreased substantially. Conjugation of sFlt to HyA has been analyzed using BCA assays and has resulted in an average efficiency of 26.3% (+/-8.15%). The ability of sFlt to bind VEGF did not depreciate once bound to hyaluronic acid, regardless of the conjugation ratio as shown in the figure below. Furthermore, unconjugated and mvsFlt have shown similar abilities for binding VEGF in *in vitro* HUVEC assays and inhibiting VEGF-dependent HUVEC survival. HUVECs incubated for 3 days at 20ng/ml VEGF showed significantly decreased rates of survival with increasing concentration of unconjugated or mvsFlt.

Conclusions: Our novel approach to of sFlt conjugation to hyaluronic acid has excellent potential for significantly increasing tissue residence time of a VEGF antagonist. Currently available techniques for treating proliferative diabetic retinopathy require up to 12 intravitreal injections of Bevacizumab or Ranibizumab per year, relying on patient compliance for effective inhibition of VEGF dependent disease progression. Our approach has the potential to strongly inhibit VEGF for longer periods of time, which can substantially reduce the number of injections required per year. We have shown that the multivalent form of sFlt is equally capable of inhibiting VEGF activity, and we anticipate that tethering the antagonist to a larger biopolymer will significantly reduce the rate of clearance from the vitreous, which overcomes a substantial obstacle for increasing the duration of effective anti-VEGF therapy.

References:

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