Cartilage-Targeting Cationic Carriers for Sustained Delivery of Interleukin-1 Receptor Antagonist in a Rabbit Osteoarthritis Model

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Introduction: Preclinical OA therapies for the knee, although mechanistically promising, have failed translation to the clinic. One reason is the lack of joint bioavailability even after an intra-articular (IA) injection due to continuous synovial fluid turnover in the joint (therapeutic half-life: ~4hr[1]) and the avascularity, dense extracellular matrix and the high negative fixed charge density of cartilage that hinders the transport of most macromolecules. To directly address these limitations, we have designed cartilage-targeting cationic drug delivery peptide carriers (CPCs) that transport rapidly within the anionic extracellular cartilage space in high concentrations after local injection, offering long-term intra-cartilage drug depot retention[2,3]. We previously used click chemistry to conjugate IL-1 Receptor Antagonist (IL-1RA) to an arginine-rich CPC motif with net charge of +14 (IL-1RA-CPC) and demonstrated that it outperforms free IL-1RA in preventing IL-1-induced inflammation in bovine cartilage explants over 16- day culture period with only a one-time dose[4]. Here we design a novel cationic fusion protein, IL-1RA-CPC that is combined into a single amino acid sequence using computational modeling such that it can maximally bind to the IL-1 Receptor 1 (IL-1R1). Moreover, we investigate the *in vivo* pharmacokinetics of CPCs in both healthy and post-ACL transected (ACLT) rabbit knees at 1- or 7- days following injection. CPC motifs offer enhanced uptake and half-life of biologics in the knee joint and may assist OA therapies towards translation.

Methods: Cationic peptides with net charge of +14 (3.6kDa: RRRRNNRRRNNRRRNNRRRR) were synthesized with a Cy5 dye at the N-terminal to track tissue biodistribution. Right knees of 12 mo. old female New Zealand White Rabbits underwent ACL transection surgery and the left knees were used as sham surgical controls. After 24h, both knees were IA injected with 500uL of 300μM CPCs; N=6 animals were sacrificed at day 1 and N=6 were sacrificed at day 7 following IA administration. All joint tissues including cartilage, meniscus and other soft tissues were harvested and imaged using a confocal microscope. Tissues were later digested, and CPC concentration was measured using a plate reader. In another set, both knees underwent either ACLT or sham surgery (N=5 each condition) and were IA injected with labeled CPCs. Blood samples were collected at 0.5, 2, 6, 24, 48 hr from the lateral ear vein to measure extra-articular kinetics. Cartilage tissue fixation and decalcification was performed starting 2 weeks post-surgery and cartilage tissue was stained for proteolytic markers. The top performing protein modeling DI-TASSER + AlphaFold 2 software was used to fold IL-1RA-CPC variants with different linker types and attachment locations (**Fig. 1A**). This includes CPC attached to IL-1RA at the N-terminal, C-terminal, C-terminal with a flexible linker (GGGGSGGGGS), or C-terminal with a hybrid linker (EAAAKGGGGS). Each folded mutant was docked to a prepared IL-1R1 structure (PDB: 1IRA) with Maestro Schrödinger PIPER docking[5], and the top representative pose based on energy scoring was chosen. Each complex, including the native IL-1RA-IL-1R1 complex (PDB: 1IRA), was simulated in a 0.15M NaCl water box for 50 ns with the OPLS4 force field. ΔG bind was estimated from each frame 0.5 ns apart from 40-50ns of the simulation trajectory with a MM/GBSA script.

Results: Single dose of IL-1RA (5000ng/mL) only when conjugated with CPC via a Maleimide-PEG-NHS Ester linker prevented IL-1 induced cell death in cartilage explants over 16 days **(Fig. 1B)**; unmodified IL-1RA could not rescue chondrocyte death. Through protein-protein molecular docking to IL-1R1 and subsequent molecular dynamics simulations, we found that the CPC sequence is best suited when added at the C terminus of IL-1RA **(Fig. 1C)**. Moreover, we found that when a hybrid, but not flexible, linker was added between the IL-1RA C terminus and CPC sequence, binding affinity to IL-1R1 was unaffected compared to the native IL-1RA-IL-1R1 crystal structure. The retention of CPCs within the joint 1 and 7 days after IA injection was observed with the bright blue color covering the tibial plateau. Cy5-CPCs penetrated at least 70μm from the superficial to deep zone (SZ - DZ) below the cartilage surface in both sham and ACLT knees **(Fig. 2B)**. Full quantification of CPC uptake in various cartilage joint tissues revealed timepoint and ACLT surgery resulted in statistically significant decrease in CPC concentration in the joint **(Fig. 2C)**. In an ACLT environment, early stage proteolytic degradation of GAGs was evident through SafO, MMP-13, and NITEGE staining 2 weeks post-surgery (**Figure 2D**) Moreover, we observed a statistically significant increase in vascular clearance of CPCs at 30 minutes following injection in rabbits with ACLT surgery **(Fig. 2E)**. From 1 day to 7 days following injection, we observed a 2.1x decrease of Cy5-CPC retention in Sham rabbit knees compared to a 2.5x decrease in ACLT.

Discussion: The intra-joint bioavailability of IL-1RA can be significantly enhanced with cationic peptide carriers (CPCs). After injection into the joint space, CPCs infiltrate into cartilage tissues and retain for at least 1 week in healthy or ACLT knees. We have designed a single sequence human recombinant IL-1RA-CPC that we predict will have effective binding to IL-1R1 and downstream therapeutic benefit in a rabbit ACLT animal model. We suspect upregulation of proteolytic activity induced by ACL transection, indicated by histology, may undesireably degrade CPCs, thus explaining increased blood clearance. Future work involves modifying the CPC sequence to enhance its proteolytic stability and evaluating IL-1RA-CPC's transport in cartilage.

Significance/Clinical Relevance: Our encouraging results demonstrate cationic peptide carriers (CPCs) have effective transport and retention in post-ACL transection rabbit knees and can offer prolonged bioavailability of therapeutics such as IL-1RA and other anti-catabolic/pro-anabolic biologics.

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References: [1]Chevalier+ *A&R* 2009; [2,3]Vedadghavami+ *Acta Biom.* 2019/2022 [4]Mehta+ *OAC* 2023, [5]Kozakov+ *Proteins: Struc., Func., Bioinfo.* 2010

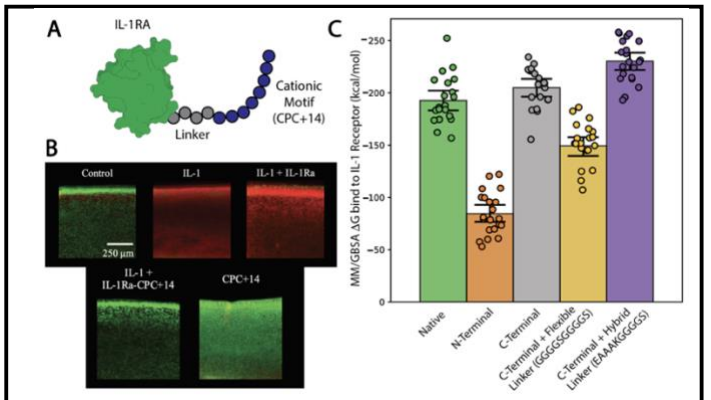

Figure 1: A) Schematic of IL-1RA covalently attached to cationic peptide carrier (CPC) motif for intra-cartilage transport and retention. **B)** IL-1 treated bovine cartilage explants given a single administration of IL-1RA-CPC+14 (with Mal-PEG-NHS Ester linker) (5000ng/mL) but not IL-1RA alone (5000ng/mL) prevents cell death (red = propidium iodide/dead cells; green = fluorescein diacetate/live cells). Figure from our previous work[4] **C)** Positioning of cationic motif and linker type onto IL-1RA affects the computational binding free energy (MM/GBSA) of IL-1RA-CPC to its receptor (IL-1R1) at snapshots throughout molecular dynamics simulation.

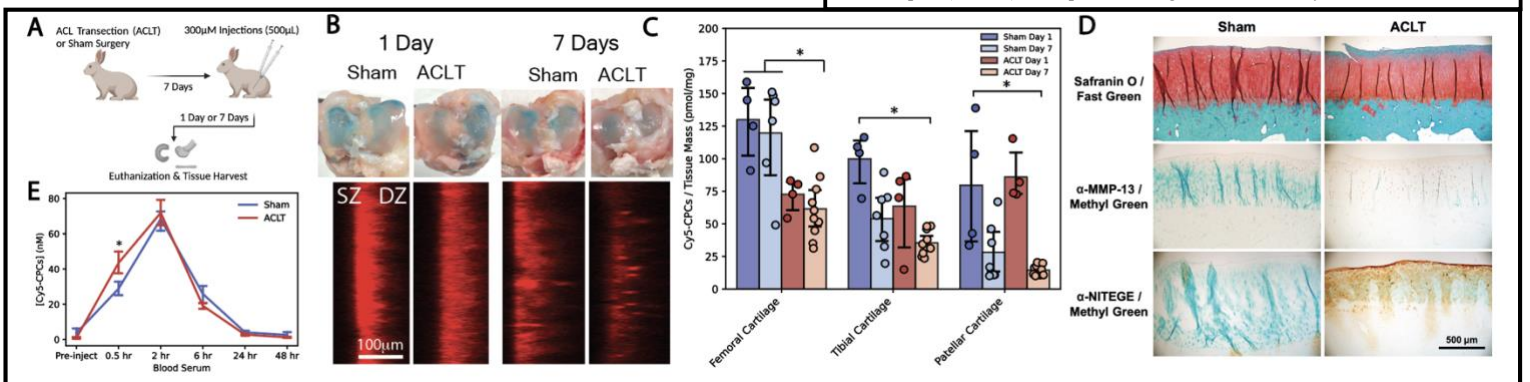

Figure 2: A) Timeline of surgery and injections of Cy5-CPCs in sham surgery or ACLT rabbit knees. **B)** Penetration and retention of Cy5-CPCs in tibial cartilage with confocal microscopy. **C)** Uptake of Cy5-CPCs in cartilage tissues following tissue digestion and quantification. **D)** Histological imaging of GAGs and proteolytic markers 2 weeks post-surgery. **E)** Vascular clearance of Cy5-CPCs in blood serum at different timepoints. A p-value < 0.05 was determined to be significant as determined by Kruskal-Wallis ANOVA and Dunn's MC Test.