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PRINCIPAL INVESTIGATOR: Dominik R. Haudenschild

CONTRACTING ORGANIZATION: The Regents of the University of California (Davis)
Sacramento, CA

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Dominik R. Haudenschild

E-Mail: drhaudenschild@ucdavis.edu

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University of California, Davis
4635 Second Avenue
Sacramento CA 95817

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The majority of common joint injuries (~900K/yr) ultimately progress to osteoarthritis (OA). As of today, there is no way to prevent PTOA after joint injury. Pathogenesis of PTOA starts with a joint injury, which then rapidly establishes a highly catabolic and inflammatory environment inside the joint. This highly catabolic inflammatory environment is itself a "secondary injury" that irreversibly degrades joint tissues and greatly accelerates OA. This secondary injury is not addressed by current clinical management of joint injuries. We advocate for a paradigm shift to treat joint injuries promptly and prevent this secondary injury. Our central hypothesis is that PTOA is preventable by promptly limiting the cellular injury responses that create this highly catabolic inflammatory environment. Preliminary data supported this approach in mouse and rat models with systemic administration of our therapeutic agent, a kinase inhibitor directed at Cdk9. In this TTDA we formulate the Cdk9 inhibitor for sustained and local delivery into the injured joint, and test efficacy in a larger animal sheep model of post-traumatic osteoarthritis. The goal is an IND-enabling study, with the intent to commercialize the product or license it for commercialization.

COVID-19-related mandatory shutdowns delayed our progress. Despite these shutdowns, I'm excited to report that we made significant progress in formulating the compound, with two candidate formulations identified. We've nearly completed the in-vitro release studies, and initiated stability studies on these formulations. We established and validated methods for detecting API in plasma and synovial fluid of small-animal and large-animal pre-clinical models. We initiated the first set of PK studies in sheep. We have a contract with a US-based API supplier that can produce GMP grade material at scale. We increased our IP position with new provisional and continuation patent filings. We further strengthened the foundation for our IP position through bio-informatics analysis (this could be done remotely during COVID shutdown) using existing scRNASeq data from a mouse PTOA model. We outlined a clinical trials strategy for first-in-man studies. Finally, we initiated a pre-IND meeting with the FDA, which is scheduled for mid-April of 2021.

15. SUBJECT TERMS

Post-traumatic Osteoarthritis, Cdk9 inhibitor, Intra-articular injection, sustained-release formulation

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Contents

Introduction

Almost all common joint injuries, especially among military personnel, will become arthritic over 2-10 years. Arthritis poses a major health problem due to severe pain that limits mobility, which leads to obesity, cardio-vascular diseases, diabetes, depression, and eventually an overall decline in health. Based on our latest findings of the underlying causes of PTOA at the molecular and cellular levels, we believe that we can develop a simple and side-effect free intervention to prevent this type of arthritis, by delivering a single shot of our proposed medicine into the injured joint.

Current clinical treatments of common joint injuries only focus on treating symptoms such as pain, and therefore, do little to stop or slow down the harmful effects of the ensuing inflammatory injury response, which further damages joint tissues and causes PTOA. Our body reacts to injury by mounting an immediate and very strong acute injury response at the molecular and cellular levels, which occurs within the first hours to days after an injury, and directly contributes to PTOA development within 2-10 years. Therefore, our team's novel therapeutic strategy is to stop the initial injury response before it can damage the joint tissue.

Our proposed PTOA preventive medicine contains two components. The active drug component Flavopiridol is an FDA-designated "orphan drug" that is a small-molecule Cdk9 inhibitor proven by us to inhibit acute injury responses and prevent PTOA in animal models. The second component consists of a drug delivery vehicle that slowly releases the active component over several weeks to provide continued protection. This delivery vehicle has also just received FDA-approval as a carrier for another steroid drug that relief pain/inflammation in arthritic patients. Using our proposed medicine, we have successfully prevented PTOA after joint injury in mouse and rat models. Our drug formulation shows multiple benefits at the cellular level, including less cell death, inflammation, and joint tissue damage.

In this proposal, we plan to test our PTOA prevention strategy in a pre-clinical large-animal model (sheep), to bring our drug formulation a giant step closer to human clinical trials. The primary outcome to be measured is preventing or significantly reducing PTOA after joint injury in sheep over the long term (18 months). Secondary outcomes include reduction of short term symptoms associated with joint injury, such as inflammation, pain, and early osteoarthritis markers. Much research and development effort is also envisioned for tuning the drug formulation and delivery vehicles to achieve those outcomes.

The short-term impacts of this work include: (a) to achieve a sustained-release drug formulation of an existing FDA approved drug that offers long term symptomatic relief of pain and inflammation after joint injury in a single injection; (b) to prevent PTOA after joint injury in a pre-clinical large animal model; and (c) to attain critical information needed to fine tune formulation of the proposed therapeutics in preparation for future human clinical trials. The long-term impact is to revolutionize the current clinical practice of the "wait-and-see" approach in managing common joint injuries, which we argue should be treated promptly, in order to prevent secondary joint tissue damage and the development of PTOA. This new therapeutic strategy shall improve the quality of life for our military personnel, as well as the large numbers of civilians who suffer joint injuries.

Keywords

Post-traumatic Osteoarthritis

Cdk9 inhibitor

Flavopiridol

PLGA Poly(lactic-co-glycolic acid)

Sustained-release formulation

Intra-articular injection

Accomplishments

Note: New information in this report will be in blue-shaded text.

COVID-19 introduced significant delays. California, and UC Davis, had mandated shutdowns. For about six months the labs were completely shut down and all in-person work was prohibited. After six months, our labs were allowed to operate at 25% capacity, then at 50% capacity. During this time many core facilities and animal facilities were unavailable to us, and supply delivery was unreliable. Even today (March 2021) I am limited to 66% capacity in the laboratories at UC Davis.

To adapt to this new reality, we devised related projects that could be performed with existing data from home. This included bio-informatics-based analysis of single-cell RNA Seq data that we had previously generated using a mouse ACL-rupture model of PTOA. This data analysis helps strengthen our patent position by generating additional insight into how Cdk9 inhibitors affect early OA.

At the same time, Tesio Pharmaceuticals was not nearly as limited by these COVID restrictions as our laboratory at UC Davis. At Tesio, we did a tremendous amount of planning and coordinating over countless hours on Zoom and also in-person. This includes establishing and testing a gel-based formulation that has several advantages over microparticle-based formulations, new patent applications filed jointly with UC Davis, and the finalized license agreement between Tesio and UC Davis. Perhaps the most significant outcome this year is that we have a pre-IND Package thta includes an outline of our proposed clinical trials strategy, and a pre-IND meeting with the FDA that is scheduled for mid-April 2021. With the recruitment of our CEO and Chairman, the Tesio team grew to include experts in IP Strategy, Corporate Development, CMC, Regulatory, Manufacturing, Formulations, Pharmacokinetics, Toxicology, FDA Compliance, and more.

Summary: Year 1 of this 3-year project was dedicated to developing a sustained-release formulation of the Cdk9 inhibitor Flavopiridol, by encapsulating the compound in PLGA microspheres. The formulation design parameters included linear sustained-release of approximately 3μg Flavopiridol per day for approximately 4 weeks, with a particle size 15-20μm in diameter. This goal was achieved.

Furthermore, the formulation was transferred to our Pharmaceutical company, Tesio Pharmaceuticals Inc. Tesio, at their expense, contracted with a third-party CRO to develop a commercially viable manufacturing process based on this formulation. This required increasing the process scale from a few milligrams to 5-10g, and increasing the robustness and reproducibility of the process. The same design parameters were met, and a joint UC Davis-Tesio patent was filed for the formulation (see below). Tesio contracted and paid for the sterile-manufacture of a batch of prototype doses, including endotoxin and sterility testing.

Tesio, at their expense, established *in-situ* forming PLGA gel formulations for the sustained-release delivery of Flavopiridol. There are many advantages of an *in-situ* gelling product over a microparticle formulation from the standpoint of commercialization. These advantages include the ease of rapidly testing multiple formulations during development, lower manufacturing costs, greater control over the supply chain, ease of final sterilization. Tesio and UC Davis are jointly filing patents on the gel formulations, and Tesio has transferred the gel material and know-how to UC Davis for further development within the scope of this award.

▷ *What were the major goals of the project?*

The major goals of the project as stated in the SOW were:

Aim 1 - Establish a formulation of Sustained-Release Cdk9 Inhibitors for Intra-Articular Injection

Major Task 1: Develop 3 Formulations with varying in-vitro release kinetics

Sub-task 1: Generate Microparticle Formulation and Characterize *in-vitro* Release Kinetics, Cytotoxicity, and Bioactivity.

Milestone achieved: We accomplished this sub-task in late 2019. This was done in coordination with Tesio Pharmaceuticals, which we founded as part of this TTDA application. The laboratories of co-Investigators Jamal Lewis and Gang-yu Liu established the formulation conditions and parameters that yielded the desired release kinetics (4-week linear sustained release), particle size (15-20 microns in average diameter), drug loading (between 1 and 1.5% flavopiridol), and release kinetics (3μg/day). Several different formulations were tested with different *in-vitro* release kinetics. The formulation with 4-week release kinetics was chosen for further development and characterization. Once the desired parameters were established and characterized in the UC Davis laboratories, the formulation was transferred to Tesio Pharmaceuticals. Tesio contracted further development of the formulation to Massachusetts-based Phosphorex Inc, a contract research organization (CRO) that specializes in drug-encapsulation for sustained-release.

At Tesio, we developed a gel-based extended release formulation. In this gel formulation, Flavopiridol is in a viscous solution that contains PLGA in a bio-compatible hydrophobic solvent. Upon injection into an aqueous environment such as the joint space, a gel forms almost immediately. The Flavopiridol is released from the PLGA gel over an extended time that is determined by the properties of the solvent and the PLGA. Tesio designed the release kinetics of the gel to be similar to the microparticle formulation, with sustained-release of approximately 3μg/day for at least 4 weeks.

Milestone achieved: We have a successfully formulated PLGA-encapsulated Flavopiridol with the desired release kinetics *in-vitro*. At Tesio's expense, Phosphorex was contracted in 2017-18 to increase the scale and develop a more robust, reproducible, and commercially viable process. A batch of this formulation was ordered and is scheduled for delivery Q2 2020 for use in the ovine surgically-induced osteoarthritis. The batch will be manufactured under sterile conditions, sterile-filled into single dose glass containers, lyophilized, and samples tested for endotoxin and bio-burden (sterility) before being injected into animals. This was outsourced to Phosphorex because sterile manufacture and filling are not within the capabilities our University of California academic research laboratories.

Tesio manufactured sterile batches of PLGA-gel formulations for animal testing at UC Davis.

Sub-task 2: Stability study for Drug Formulation.
Milestone achieved: Samples of the microparticle PLGA-encapsulated Flavopiridol were set aside to perform stability studies in the future. The samples are stored at -20°C, aged approximately 30 months. A sample of un-encapsulated Flavopiridol was also set aside and similarly stored for comparison.

Stability studies on the gel formulation have been initiated using assays and documentation suitable for presentation to the FDA. The stability studies of the gel formulation were performed by Tesio, as COVID-related shutdowns made it difficult to perform at UC Davis. Our data suggest at least 3 month stability of the gel formulation at -20C. We expect that

this level of stability will be sufficient for first-in-man studies.

Sub-task 3: Regulatory IACUC/ACURO Approval.
Milestone partially achieved: IACUC was initiated, but not yet approved in part because of the complexity of the many procedures we are proposing (intra-articular injections, surgeries, repeated arthrocentesis and serum draws, X-ray, MRI, CT, gait analyses). The specific details are still being negotiated with IACUC, and we may end up splitting the protocol into smaller parts. My home department (Orthopaedic Surgery) just hired a Laboratory Manager, whose job description includes IACUC protocol development and maintenance. This should expedite the process.

We decided to separate the PK study from the much more complicated surgically-induced PTOA model in sheep. We obtained IACUC and ACURO approval for the PK study in sheep a few weeks ago. It turns out we were required to fully anesthetize the sheep for the arthrocentesis (with intubation and a ventilator). This increased the manpower required to perform even the relatively simple PK time-courses, and conflicted with COVID-related room occupancy restrictions. Now that COVID-restrictions are somewhat eased, we are able to move forward with the PK studies while working on the regulatory approval for the PTOA studies.

Major Task 2: Identify the formulation with best *in-vivo* performance.

Sub-task 4: Test three candidate formulations *in-vivo*, establish pharmacokinetics and toxicity profiles in 8-week sheep study (12 sheep)
Milestone partially achieved: IACUC and ACURO protocols were initiated but as of November 2019 we have not yet received IACUC approval for performing the animal studies. Once IACUC and ACURO approval are obtained, we are poised to start the animal studies. However, separately from the studies at UC Davis, Tesio made progress in testing the jointly developed formulation in equine patients. This includes a 6-week PK study in healthy equine joints. Although more characterization is required, early data suggest that the Tesio formulation has appropriate *in-vivo* performance and no apparent local or systemic toxicity. We don't expect large differences in the *in-vivo* performance of the formulation between horses and sheep. We propose to use this formulation for the ovine studies once IACUC/ACURO approval is obtained.

The PK study in equine joints was repeated using the PLGA microparticle formulation provided by Tesio. The results are now fully analyzed with validated methods for the detection of Flavopiridol in both equine plasma and equine synovial fluid. The Tesio microparticle formulation has appropriate in-vivo performance, and no apparent local or systemic toxicity was observed. The systemic burden of Flavopiridol in horses was negligible, with plasma levels below the lower limit of quantification at all time points. The synovial fluid concentrations were lower than expected, but the duration of the release was appropriate. We interpret this data to indicate that the pharmacokinetics of the microparticle formulation is suitable, although the dosing still needs to be further optimized.

To be very clear, these equine studies were *not* performed with PR171305 funds. The funds for these equine studies were from an internal grant from the UC Davis Center for Equine Health (CEH) received by Co-Investigator Derek Cissell. The equine studies used the same microparticle formulation provided by Tesio, so they contribute to the overall goal of commercializing the extended-release flavopiridol product. This is why I am including

the equine data in this report.

We developed and validated methods to quantify Flavopiridol in horse plasma and horse synovial fluid. This was cost-effective because both these fluids are readily available from equine patients at the UC Davis School of Veterinary Medicine. Typical yields of synovial fluid from the healthy equine carpal joints was nearly 5ml. Human synovial fluid is difficult to obtain with concerns about patient safety, confidentiality, consent, especially for healthy patients. In sheep, the typical yield of synovial fluid is under 1cc, insufficient for methods development. The method development work for determining Flavopiridol in equine synovial fluid serves a template for methods in sheep, rat, and human synovial fluids.

We developed and validated a method to quantify Flavopiridol in sheep plasma. The method for quantifying Flavopiridol in sheep synovial fluid is not yet validated, this is ongoing.

Summary: Aim 1 is nearly complete. We identified a formulation of PLGA-encapsulated Flavopiridol that has the desired in-vitro characteristics, and with good in-vivo *performance at least in equine subjects. We developed a PLGA-gel formulation with major advantages over the microparticle formulation. We developed and validated methods for detecting Flavopiridol in plasma from multiple species including sheep, and in synovial fluid. Remaining to be done in Aim 1 are the PK/PD studies in ovine synovial fluid and blood, and the determination of local and systemic toxicities in ovine joints.*

Aim 2: Test Efficacy in a Sheep Model of Post-Traumatic Osteoarthritis

This section was impacted by COVID-related delays, and also by developments in the animal models used to test osteoarthritis therapeutics. We propose to change the scope of Aim 2 to accommodate these unexpected delays. First, the 18-month time point is no longer feasible even with a 1-year no-cost extension to the project. At the same time, the field has moved away from the longer (18-month) time points, and 6 to 12-month studies are now accepted standards. Finally, the 18-month study is by far the most costly aspect of this proposal. As such, we propose to eliminate the 18-month time point, and apply the savings to cover the cost of COVID-related delays.

Major Task 1: 4-Month Sheep Study and two months for analyses.

Sub-task 1: Primary outcome is histological grading of OA in sheep stifle joint, on the 6-point OARSI scale of 0-5. Time points initially proposed were 4, 12, 18 months, (groups A, B, C). Interim analyses at 4 months to determine course of action for 12, 18 month study (whether we need to adjust drug dose).
Not Started: This is scheduled to begin in the 2nd year of the grant, and was delayed by COVID-19.

Major Task 2: 4- and 12-Month Sheep Study and analyses.

Sub-task 2: Primary outcome is histological grading of OA in sheep stifle joint, on the 6-point OARSI scale of 0-5. A second interim analysis when 12-month animals are sacrificed. Final re-analysis with all animals after the 18 month animals are sacrificed.

Not Started: This is scheduled to begin in the 2nd year of the grant, and was delayed by COVID-19.

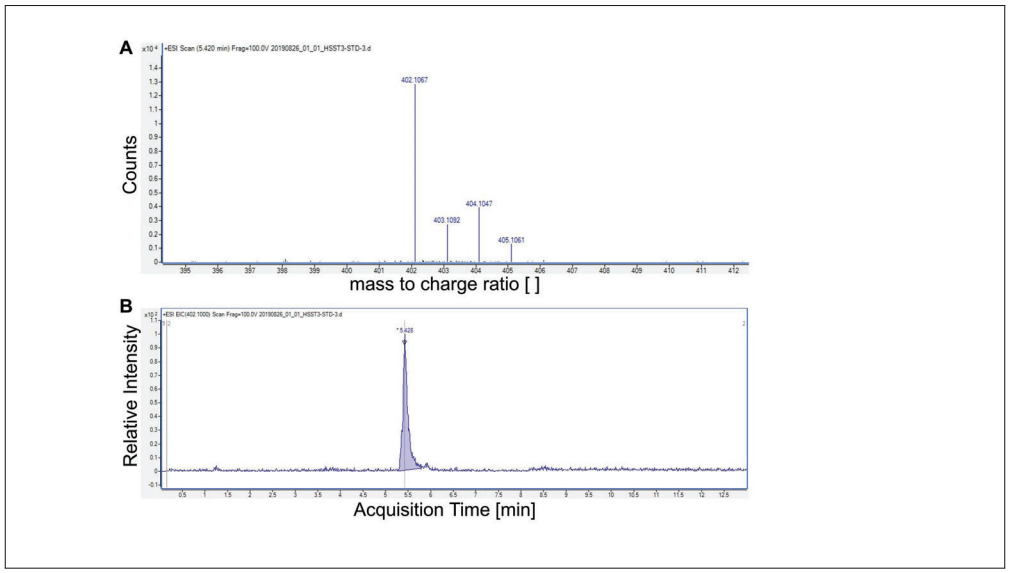

Figure 1: A) Mass spectrum of flavopiridol. B) Extracted ion chromatogram for flavopiridol mass feature from panel A.

▷ *What was accomplished under these goals?*

1. **Metabolomics analysis of Flavopiridol concentrations** (Co-Investigator Associate Professor Ron June, Ph.D. Montana State University)

   In the first year of this project, we have completed studies in support of Major Tasks 1 and 2. Working with Dr. Haudenschild's group at UC Davis, we obtained samples of flavopiridol. Our initial studies developed a metabolomics assay for detection of flavopiridol. This included finding retention times and mass adducts (Fig 1). After confirming that we could detect flavopiridol, we created standard solutions and developed a quantitative mass spectrometry assay. This assay shows that quantities as low as 50 nanomolar are detectable, and that there is good linearity above 500 nanomolar (Fig 2). We recently received samples from Dr. Haudenschild's laboratory, and we will begin using the quantitative assay to determine flavopiridol concentrations from these samples. Planned studies for the future, per discussions with Dr. Haudenschild, include global metabolomic profiling of synovial fluid from animal models of post-traumatic osteoarthritis including control- and flavopiridol-treated animals.

2. **Encapsulation of Flavopiridol in PLGA for sustained-release** (Co-Investigator Professor Gangyu Liu, Ph.D.)

   (a) **Seeking greener approach with higher degree of control over the size and structure of PLGA particles**

   The PLGA particles were frequently produced by emulsion techniques. Our team has produced our initial batches of PLGA particles using emulsion method. We identified two short comings in these conventional approached: (1) potential healthy and environmental hazard; (2) lack of control in sustained delivery behavior. First, all of currently adopted synthesis methods utilize the evaporation of volatile organic compounds (VOC) such as dichloromethane (DCM), a known carcinogen. DCM is recognized by the international coun-

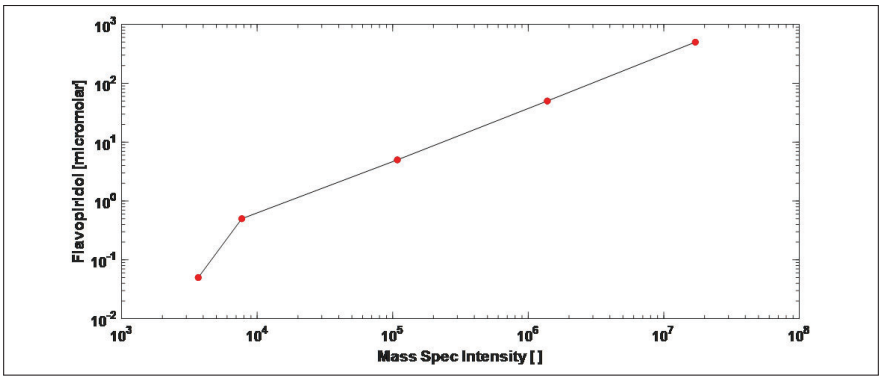

Figure 2: Calibration curve to predict Flavopiridol concentration from mass spectrometry intensity.

cil for harmonization of technical requirements for human use (ICH) as a class 2 solvent, which residual amounts in medicinal products is limited to no more than 600 ppm.4-5 Therefore, our team decide to think ahead of time to assure health and safety of our military personal, and avoid expose them to any danger due to drug delivery. In addition to human health, DCM has been in recent headlines for the ban on consumer use as imposed by the current federal government, therefore an alternative solvent is essential for safer and environmental protection agency (EPA) compliant use. Secondly, the emulsion methods, while simple and high throughput, produced polymer particles with various size, i.e. broad size distribution. As a result, it is difficult to predict and reproduce the drug release kinetics. To assure the sustained release for the proposed applications, our team decide to employ microfluidic flow focusing device to produce particle with narrow size distribution.

(b) **Synthesis of Drug Encapsulated PLGA microparticles using microfluidic flow focusing and a green organic solvent**

Our device was constructed during the first grant year, and is shown in figure 3 (page 13). The flow-focusing microfluidic were designed in AutoCAD 2018, with a 120 μm channel height and 100 μm orifice diameter. The device was fabricated using poly(dimethylsiloxane) (PDMS) soft lithography technique8 by uFluidix (Toronto, Canada) and bonding to a glass slide. The microfluidic channels were rendered hydrophilic using PVA treatment using similar methods as described in literature. Briefly, the microfluidic device underwent plasma treatment with air for 5 minutes, followed by filling the channels with aqueous 1% PVA (98% hydrolyzed) and left for 15 minutes. The PVA solution in the channels was removed by blowing N_2 gas into the channels and then placed in an oven at 110 °C for 15 minutes to remove excess PVA. This procedure was followed two more times to ensure hydrophilicity of the channels.

Flavopiridol-loaded PLGA microparticles were prepared using Flow-Focusing Microfluidics using similar methods as described previously. For 15 μm microparticles, PLGA (10 mg) was dissolved in 1 mL of 0.124 mg/mL flavopiridol/DMC solution for a desired loading of Flavopiridol:PLGA (1.22% w/w) and left to fully dissolve overnight. For 32 μm microparticles, 3.0 mg of Flavopiridol was added to 8.6 mL of DMC in a glass scintillation vial and heated to 60 °C for 1 hour to obtain a 0.35 mg/mL flavopiridol/DMC solution. 40 mg of PLGA was added to the 0.35 mg/mL solution and allowed to dissolve overnight for a desired

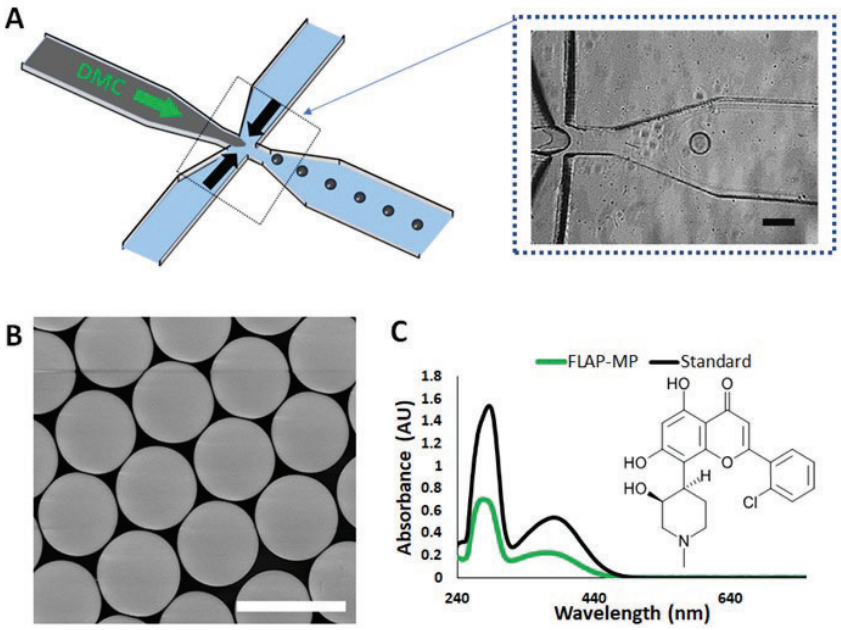

Figure 3: A) Schematic diagram of Microfluidic Flow Focusing Device, where (1) is the Organic phase region consisting of PLGA/FLAP/DMC, (2) indicates the aqueous region consisting of 1% PVA flowing in the direction of dark arrows, (3) collection channel. The zoom in of the schematic is shown to the right of (A) highlighting droplet formation in the optical image where scale bar = 100 μm. B) SEM image of 15 μm PLGA microparticles fabricated from Microfluidic synthesis and (C) absorption spectrum of dissolved flavopiridol-loaded PLGA MP's(FLAP-MP) and 100 μM Flavopiridol in DMSO. The corresponding chemical structure is of Flavopiridol. Scale bar of SEM image = 20 μm

loading of Flavopiridol:PLGA (0.87 % w/w). The organic solution was transferred into a gas-tight syringe and equipped on a syringe pump. 1% w/v poly(vinyl alcohol) (PVA) aqueous solution was prepared as described previously9 and was transferred into two gas-tight syringes and equipped on a separate dual-syringe pump. The two aqueous syringes were attached via PTFE tubing and inserted into the flow-focusing microfluidic device's aqueous channel inlets, while the organic syringe was attached to via PTFE tubing into the organic channel inlet. PTFE tubing was inserted into the outlet of the microfluidic device and the open tubing was immersed in a round bottom flask (100 mL) containing 1% w/v PVA (5 mL). Droplet formation was visualized *in-situ*, using an inverted microscope equipped with a high-speed camera (Photron AX-100). The microfluidic experiment was run from 1-7 hours, depending on desired yield, and following synthesis the collection flask was mounted on a rotational evaporator (Buchi R300) and DMC was evaporated under reduced pressure at room temperature (40 mbar, 5 minutes). Following evaporation, the PLGA microparticles were collected via centrifugation (2500 RPM, 10 min) and washed 3X with water to remove residual PVA. Following washing, the microparticles with quickly frozen in liquid nitrogen and lyophilized to dryness overnight. The flow rate of the dispersed organic phase (Q_d) was fixed at 0.1 mL/hr, while the flow rate of the continuous aqueous phase (Q_c) was varied from 2 – 6 mL/hr. The set of flow rates of the continuous aqueous phase and dispersed organic phase are indicated by $[Q_c, Q_d]$. One set of flow rates, $[Q_c, Q_o]$ = [6,0.1] and [PLGA] = 10 mg/mL and 40 mg/mL were used to obtain flavopiridol loaded microparticles with diameters of 15 μm and 32 μm. This procedure was also used for unloaded microparticles.

On the right of figure 3 (page 13), at a transient time, the optical image shows the microfluidic device dimensions and a 76 μm PLGA droplet. As shown in figure 3B, an SEM image of PLGA microparticles with diameter measured to be 15.1 $\pm$ 0.6 μm, i.e. 3.9 % dispersity. This microparticle diameter is formed using flow rates $(Q_d = 0.1\frac{ml}{hr}, ^{\circ}Q_c = 6\frac{ml}{hr})$. The chemical structure of flavopiridol is seen in figure 3C, along with the absorption spectra of free flavopiridol (black) and encapsulated flavopiridol (green) dissolved in DMSO. Two absorption maxima are shown for flavopiridol, where 274 nm is used for detection.

The dimensions of the microfluidic device are as follows: the width of inlet channels is 300 μm, the width of the outlet channel is 400 μm, 100 μm for the width of the orifice, and all channels have a height of 120 μm. The flow rates of both phases are syringe pump driven at rates that allow laminar flow as indicated by a Reynold's number (Re) less than 1.

$$Re = \frac{\rho v L}{\mu} \tag{1}$$

In the equation above, the density and dynamic viscosities of both solutions are approximated by using pure DMC11 and pure water at $\rho_{water} = 1.00\ \frac{g}{mL}$ and $\rho_{DMC} = 1.07\frac{g}{mL}$, and the dynamic viscosity at $\mu_{H_2O} = 0.891mPas$ and $\mu_{DMC} = 0.585mPas$. The mean fluid velocity (v) is approximated as the ratio of the mean flow rate (Q) and the cross-sectional area of the orifice $(A_{orifice})$.

$$v = \frac{Q}{A_{orifice}} \tag{2}$$

The hydraulic diameter (L) is calculated as the ratio of 4 times the cross-sectional area (A) divided by the wetted perimeter (P):

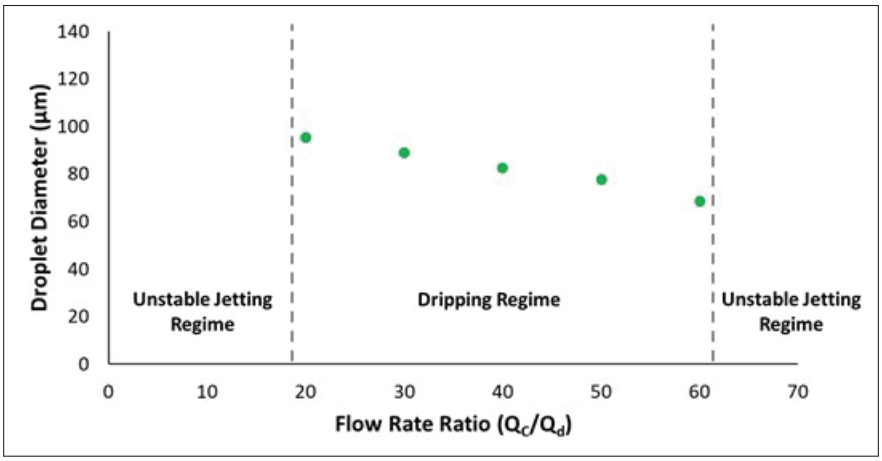

Figure 4: PLGA droplet size selection by variable aqueous flow rate and fixed [PLGA] = 10 mg/mL and organic phase flow rate = 0.1 mL/hr.

$$L = \frac{4A}{P} \tag{3}$$

The Reynolds number was found to be 0.0169, which indicates laminar flow. Once the two fluids meet at the orifice, a resulting spherical droplet is formed due to the interfacial surface tension between fluids which causes the surface area to decrease such that free energy is minimized and spontaneous. Following synthesis droplets are subject to rapid evaporation of DMC under reduced pressure using a rotational evaporator, to form a solid PLGA microparticle. 15 μm PLGA microparticles were synthesized using flow focusing microfluidics as shown in figure 4 (page 15) and characterized using scanning electron microscopy(SEM). For drug loading, flavopiridol was dissolved in DMC at 0.122% (w/v), and 10 mg of PLGA was added to 1 mL of this organic solution. Following synthesis, the verification and quantity of drug encapsulated in PLGA microparticles were accessed using UV-Visible spectroscopy by dissolving microparticles in dimethyl sulfoxide (DMSO) and comparing against a set of known standards. As shown at the bottom of figure 3C (page 13), is the absorption spectra, for which two of the three absorption maxima are seen at 274 and 380 nm before and after encapsulation. The interference of PLGA during dissolution of flavopiridol loaded microparticles was negligible and has been reported previously. The loading capacity (LC) of flavopiridol in the microparticles were accessed by determining the mass of flavopiridol after dissolution of the microparticles $(M_{loading})$ and dividing by the total mass of the microparticles (M_{tot}):

$$LC = \left(\frac{M_{loading}}{M_{tot}}\right) x100\% \tag{4}$$

The encapsulation efficiency (EE) is the amount of drug actually encapsulated (M_{loaded}) divided by the amount of drug initially desired to load $(M_{desired})$:

$$EE = \left(\frac{M_{loading}}{M_{desired}}\right) x100\% \tag{5}$$

The EE of the synthesis above was performed in triplicate and resulted in 55.0 $\pm$ 3.0 % and a final LC of 0.65 $\pm$ 0.12 %. The location of the unencapsulated flavopiridol was experimentally determined using UV-visible spectroscopy and compared against a set of standards

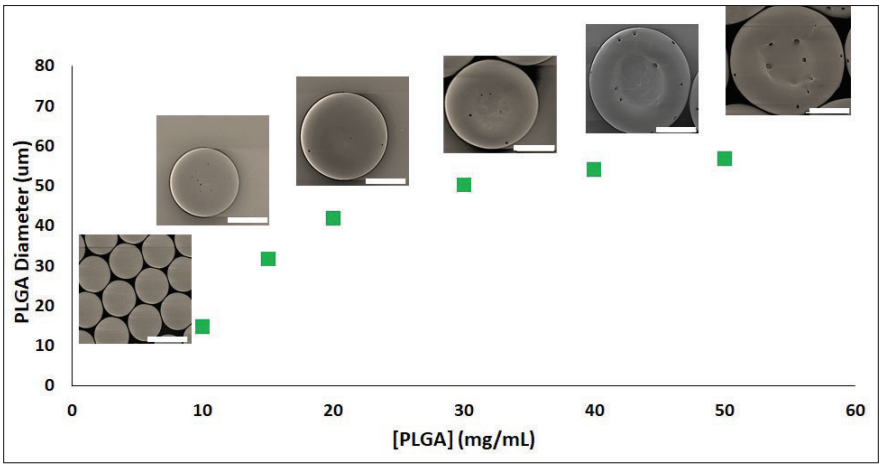

Figure 5: Microparticle diameter dependence on [PLGA] at fixed flow rates of continuous aqueous and dispersed organic phase at 6 mL/hr. and 0.1 mL/hr. Images are characterized by SEM ($V_{acc} = 2kV$). All scale bars = 20 μm

in 1% PVA to be. Following analysis, it was confirmed to be in the aqueous supernatant following synthesis. This can be explained due to the solubility of DMC in water, where during synthesis and in the collection flask DMC carried the unencapsulated flavopiridol into the aqueous phase. This successful encapsulation of flavopiridol in PLGA microparticles is the first time for both using microfluidic technology and with using a green solvent.

Flow focusing microfluidics enables microparticle size selection by variability of the aqueous flow rates by holding concentration and organic phase flow rate constant. As illustrated in figure 4 (page 15), it can be seen that two regimes can be obtained by varying the flow rate ratio, the first is the unstable jetting regime. In this regime, once the two immiscible fluids meet as seen in figure 3 (page 13), the viscous forces of the mixture competes with the interfacial surface tension between the aqueous phase and organic phase and forms a long fluid thread that pinch's off into spherical droplets in the collection channel. This unstable jetting regime produces poly dispersed droplets like those seen in bulk emulsion. In contrast, the dripping regime causes droplet formation to occur in the orifice region as seen in figure 3A. This regime produces monodispersed droplets where droplet diameter can be selected. Using these flow conditions at [PLGA] = 10 mg/mL, PLGA microparticle diameter can be synthesized from 15-35 μm.

Concentration can also be varied if all other parameters are held constant and this can be seen in figure 5 (page 16) As the concentration of PLGA increases from 10 – 50 mg/mL, the microparticle diameter increases from 15-54 μm. Flow focusing microfluidics has been shown to generate uniform particle distributions. The measure of dispersity is best seen through the coefficient of variation (CV), where the ratio between the standard deviation (σ) and the mean microparticle diameter (x). Microparticle distributions are said to be monodisperse if the CV $< 5\%$.

$$CV = \frac{\sigma}{x} x 100\% \tag{6}$$

Diameter (μm)	CV (%)	[PLGA] (mg/mL)	Q_{aq} (mL/hr)	Q_{org} (mL/hr)	LC(%)	EE(%)
15.1 ± 0.6	3.9	10	0.1	6	0.65 ± 0.12	55 .0± 3.0
32.4 ± 1.0	3.1	40	0.1	6	0.58 ± 0.02	65.2 ± 0.1

Figure 6: Properties of Flavopiridol Loaded PLGA microparticles

(c) ***In-Vitro* Drug Release Profiles**

Flavopiridol was encapsulated into PLGA microparticles using flow focusing microfluidics. Using microfluidics enabled control on flow rates and concentration with the tunability of these parameters different monodisperse diameter were chosen in order to determine the in vitro release kinetics. The goal of determining the release kinetics was for comparison against the sustained release of 300 nM in the local space of the knee joint. The microparticle size chosen for release kinetics were within the range of similar sizes and loading previously shown in bulk emulsion using DCM. Figure 6 (page 17) shows the microparticles synthesized for release and their properties.

Both microparticle batches were selected for in vitro release experiments to determine the release kinetics. The microparticles were immersed in 1X PBS with 0.2% (v/v) Tween-20 to enable a sink effect and were heated at 37 °C under mixing. At different time points in the release, microparticles were centrifuged (6000 rpm, 5 min) and the supernatant was collected and replenished with equal amount of fresh buffer. The supernatant was then analyzed using UV-Vis spectroscopy at 274 nm and compared against a series of known standards in buffer medium. The resulting profiles are shown in figure 6 (page 17) and plotted against the desired profile. The 15 and 32 μm microparticle release profiles reveal three distinct phases, all characterized with different slopes of release. For instance, the initial slope from 0-5 days is similar for both sizes, while the second phase is drastically differing the 15 μm microparticles, there is a lag phase where little drug is released for 4 days, while the larger particles quickly release 4.0 %/day. The larger particles eventually plateau at 84 % released after 31 days of release, while the 15 μm microparticles continue to release. In comparison to the desired release profile, which release near 2.5%/day, linear least square fitting of the 15 μm microparticles release 2.54 %/day for a $R^2 = 0.98$. In molar concentration, the desired release requires a slope of 2495 nM/day. The 15 μm microparticles released 3490 nM/day, for 4 mg of microparticles. Thus, to accommodate a decrease in concentration, 2.8 mg of microparticles should be used. In comparison to previous work using bulk emulsion, our greener synthesis generates release kinetics close to the desired release for at least three weeks, which is suitable for sustained release and treatment of PTOA. Work in progress to increase throughput and performing toxicity tests, and *in-vivo* efficacy measurements in animal models.

(d) **Development of new methodology for PLGA particle characterization**

We are planning to develop a QCM system integrated with the state-of-art AFM (CypherE from Oxford Instrument) for simultaneous AFM imaging while QCM monitoring degradation of composite hydrogel materials, as illustrated in figure 8 (page 19). We are planning to manufacture the liquid cell from Ti-alloy to have better bio-compatibility and thermal conductivity. The system will have liquid input, output, temperature sensor and heater to keep

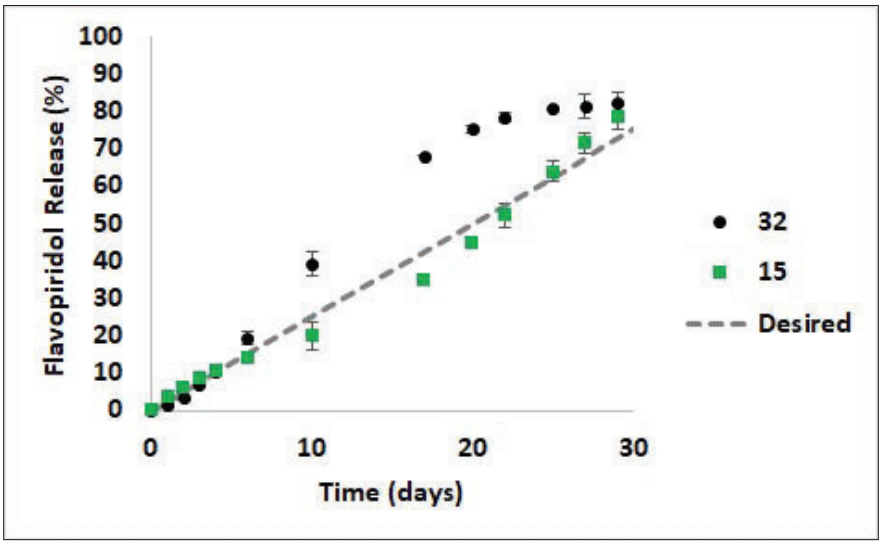

Figure 7: *In vitro* release profiles of 15 (LC = 0.65%) and 32 (LC = 0.58%) μm microparticles as compared to desired release profile (i.e. maintaining 300 nM in local knee joint). Release conditions: 1X. PBS/ 0.2% Tween-20 at 37 $^\circ$ under 100 rpm. Mass of PLGA MPs = 4 mg/0.5 mL of buffer. Release profiles are defined as the average $\pm$ s.d. for three trials.

stable the sensor temperature for temperature depended experiments and better frequency resolution. The base of QCM head will be machined from PEEK to have less thermal drift that will also hold the magnets to hold the AFM XY Scanner. Our preliminary feasibility calculations indicate that QCM and AFM can operate in the proposed configuration. QCM sensor vibrates laterally at the sensor resonance frequency in MHz range. AFM cantilever vibrates vertically at the cantilever resonance frequency around 100 kHz in liquid, thus has little interference. AFM and QCM lock-in amplifiers locks its own base frequency, further assure independence.

The current-state-of-the-art QCM sensor consists of a quartz crystal with electrodes deposited on both the front and back sides. Roughness of quartz crystal changes the frequency shift and dissipation factor of a quartz sensor. Liquid trapped into the pores and grooves causes inertial effect and liquid behave as coupled mass on the sensor surface. We plan to develop our own sensors using blank AT-cut quartz wafer with further polishing to at least optical grade average roughness, MSR 1 nm, as surface roughness also generates non-laminar liquid motion on the sensor that increase the energy dissipation and decrease Q-factor. Surface modification may be necessary to accommodate our project needs, taking advantage of the team's extensive surface chemistry experience. We hope to complete the acquisition of AFM, development of QCM and integration in year 2, taken advantages of team's expertise in scanning probe microscopy (SPM) instrumentation.

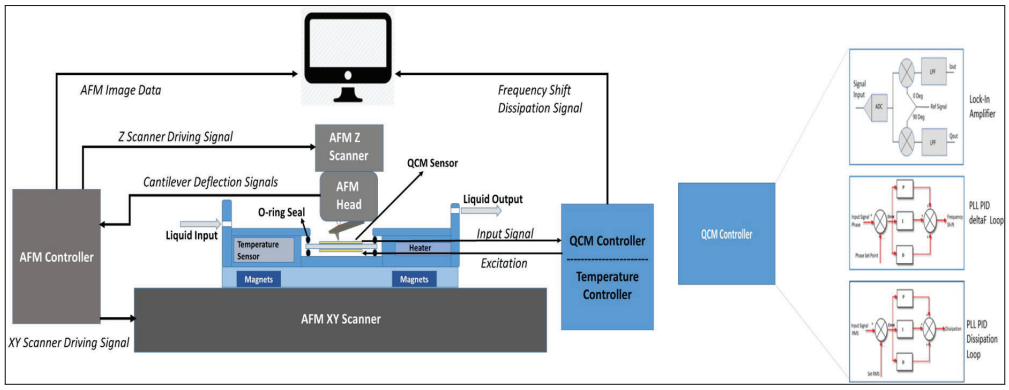

Figure 8: QCM and AFM integration block diagram, as well as the design of controller.

3. **Cdk9 Inhibitor Encapsulation** (Co-Investigator Associate Professor Jamal Lewis, PhD)
For commercialization purposes, we need to establish broad patent protection for intra-articular injections of sustained-release Ckd9 inhibitors. The patent office granted us one USE patent (9,498,471), but the patent office limited our claims to only one Cdk9 inhibitor (Flavopiridol). We needed to expand this to include additional small-molecule Cdk9 inhibitors to provide adequate IP protection. We filed a FORMULATIONS patent (provisional patent filed 2018, PCT patent filed 2019) that covers polymer-encapsulated Cdk9 inhibitors. To provide data for this patent, Dr. Lewis encapsulated various small-molecule Cdk9 inhibitors in multiple different polymers suitable for sustained-release administration. A description of the development and *in-vitro*/*in-vivo* characterization of these formulations is described in the next sections.

   (a) **Target Polymers and Cdk9 Inhibitors**
   We considered several choices of Cdk9 inhibitors and encapsulation polymers. For the Cdk9 inhibitors, we chose 4 different small-molecule inhibitors that have been tested in either Phase I or Phase II clinical trials to ensure that the inhibitors had an adequate safety profile. Furthermore, we chose 4 inhibitors that belonged to different molecular classes to prevent the patent office from limiting our claims to just one class of inhibitors. For the polymers, we chose polymers that were commercially available at reasonable prices, and that were already FDA-approved for use in humans. Figure 9 (page 19) shows the molecular structures of the Cdk9 inhibitors (Flavopiridol, Dinaciclib, SNS-032, and Voruciclib) and the polymers.

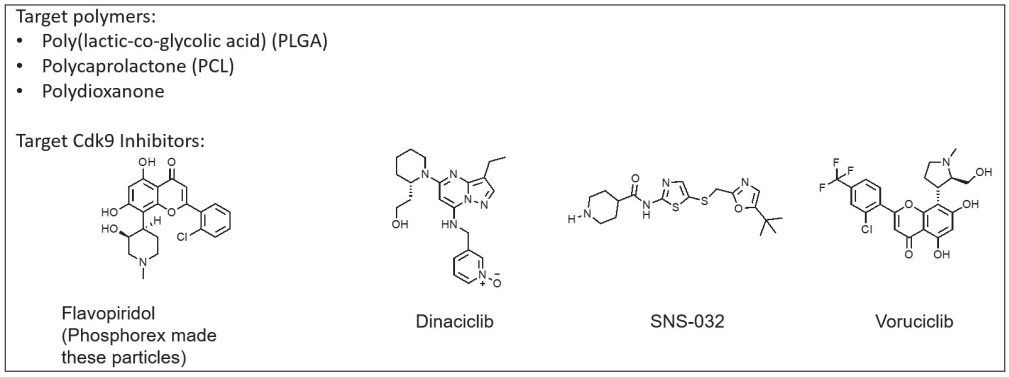

Figure 9: Target encapsulation polymers and molecular structure of four small-molecule Ckd9 inhibitors identified for sustained-release formulation

   (b) **Fabrication of polymers with small-molecule Ckd9 inhibitors** Fabrications of poly(lactic-

A) % Loading efficiency

Chemicals	with DCM/DMSO % loading		with $CHCl_3$/DMSO % loading		with $CHCl_3$ % loading	
	PLGA	PCL	PLGA	PCL	PLGA	PCL
Dinaciclib	3.6	0.6	-	0.8	3.8	7
SNS-032	30.7	0.3	-	0.2	-	-
Vorociclib	59.9	1.1	-	2	-	-

B) % Release kinetic

day	PLGA with DCM/DMSO (% release)		
	Dinaciclib	SNS-032	Vorociclib
1	0.6	4.0	3.4
3	1.5	5.4	4.8
5	2.2	7.0	6.5

day	PCL with $CHCl_3$/DMSO (% release)		
	Dinaciclib	SNS-032	Vorociclib
3	0	0	0.36
7	0	0	0.43

C) Size of microparticles using Accusizer

with DCM/DMSO

Polymers	Blank	Dinaciclib	SNS-032	Voruciclib
PLGA	17.1	9.0	16.9	13.6
PCL	9.6	8.2	7.6	11.7

with CHCl3/DMSO

Polymers	Blank	Dinaciclib	SNS-032	Voruciclib
PLGA	18.8			
PCL	14.1			

Standard deviations = 10-15 microns

Figure 10: Target encapsulation polymers and molecular structure of four small-molecule Ckd9 inhibitors identified for sustained-release formulation

co-glycolic) acid (PLGA) or polycaprolactone (PCL) particles with CDK9 inhibitors were performed using a single emulsion-solvent evaporation technique. Briefly, 500 mg of PLGA or PCL was dissolved in 2.5 mL of 2 % v/v dimethylsulfoxide in methylene chloride (for PLGA) or chloroform (for PCL). CDK9 inhibitors (2% g/g polymer, Dinaciclib, SNS-032, and Voruciclib) were added into the polymer solution, and then added to 5 mL of 10% poly(vinyl alcohol) solution in water. The solutions were vortexed at a full speed for 30 s for PLGA or 45 s for PCL to form particles. The particle suspensions were transferred to 150 mL of 1% poly(vinyl alcohol) and the solutions were stirred for 24 h. The particles were pelleted, subsequently washed with water, lyophilized and stored at -20 °C for further use.

(c) **Characterization of polymers with small-molecule Ckd9 inhibitors**
Size distribution was measured by a particle sizing systems model 770 Accusizer Optical Particle Sizer. The loading efficiency was determined as a percent of the total inhibitor remaining in the particles after formulation. *In-vitro* release kinetics were measured using UV-Visible spectrometry. We did not repeat the encapsulation of Flavopiridol in PLGA polymer as this was already performed by Phosphorex (see above). A summary of the characterization performed to date is provided in figure 10 (page 20).

(d) **Characterization of Cdk9 inhibitor activity after polymer encapsulation**
To ensure that the inhibitor activity survived the encapsulation process we performed a

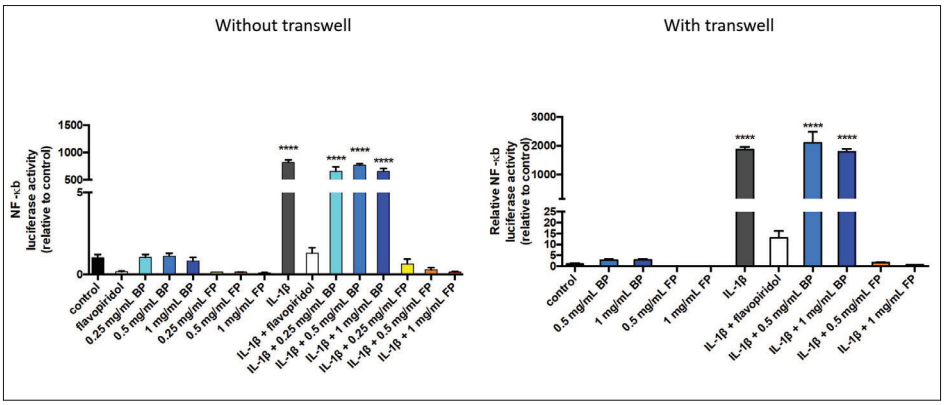

Figure 11: Cdk9 inhibitors retain their biological activity after the microparticle fabrication process

bioassay using a reporter cell line and lactate dehydrogenase assays, respectively. Briefly, the reporter cell line has a synthetic primary response gene promoter (in this case an NFκB-dependent promoter) driving the expression of firefly luciferase. The promoter is activated by adding 10 ng/ml IL-1β to culture media, which results in a Cdk9-dependent upregulation of luciferase activity on the order of 1000-2000-fold over baseline. In the presence of active Cdk9 inhibitor, the promoter is not activated by IL-1β and luciferase activity remains at baseline. The assay was performed by adding the microparticles directly onto a monolayer of cells, and also by adding the microparticles into a transwell such that the Cdk9 inhibitor was released into the culture media but without physical contact between cells and microparticles. A summary of these results is shown in figure 11 (page 21), demonstrating that Cdk9 inhibitor activity was as strong after encapsulation as before.

(e) **Characterization of cytotoxicity after polymer encapsulation**
To ensure that no cytotoxic byproducts were introduced during the microparticle fabrication process, we performed a lactate-dehydrogenase assay that quantifies the number of lysed or dying cells. As described above, this assay was performed with the microparticles added directly to the cell monolayer, and again with the microparticles added into a transwell so that the Cdk9 inhibitor was released into the culture media but without direct contact between the cells and the microparticles. The results are summarized in figure 12 (page 22), and demonstrate that the microparticle fabrication process did not increase cytotoxicity above baseline.

(f) **Characterization of Flavopiridol release kinetics *in-vitro* after polymer encapsulation**
We tested the *in-vitro* release kinetics of the encapsulated Flavopiridol using synovial fluid. Briefly, 4mg of microparticles were suspended in 500 μl of synovial fluid and stored at 37 °C for 4 weeks. At designated times, a sample of the synovial fluid was removed for analysis by mass spectrometry to assay the soluble Flavopiridol. The results are summarized in figure 13 (page 22),and demonstrate a rather large initial burst (20%) within the first 3 days, followed by a sustained release over the next 15 days. These results will need to be repeated with more technical replicates, using a larger volume of synovial fluid, and with complete replacing of the synovial fluid at regular time intervals. These measurements have now been repeated, with the results shown in figure 14 (page 22).

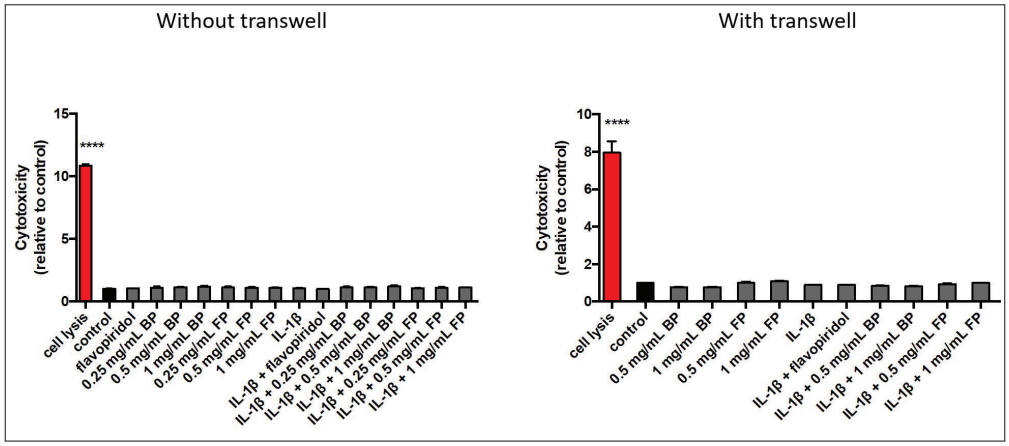

Figure 12: Lactate Dehydrogenase Assay demonstrates that microparticle fabrication did not introduce cytotoxicity

Release kinetic of Flavopiridol particles in synovial fluids

days	% release
3	20
7	23
14	32
28	33

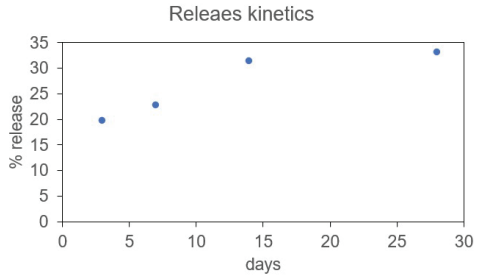

Figure 13: *In-vitro* release kinetics of Flavopiridol in synovial fluid

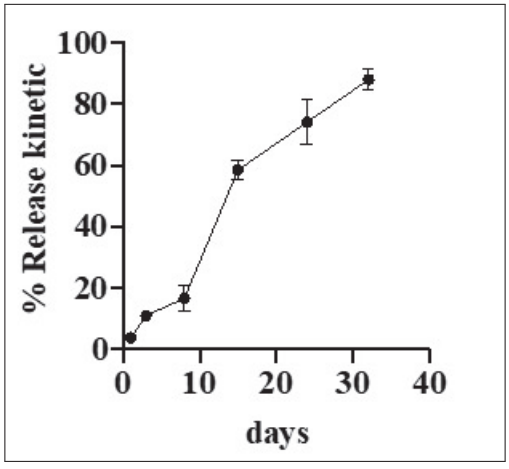

Figure 14: *In-vitro* release kinetics of Flavopiridol in synovial fluid, repeated measures

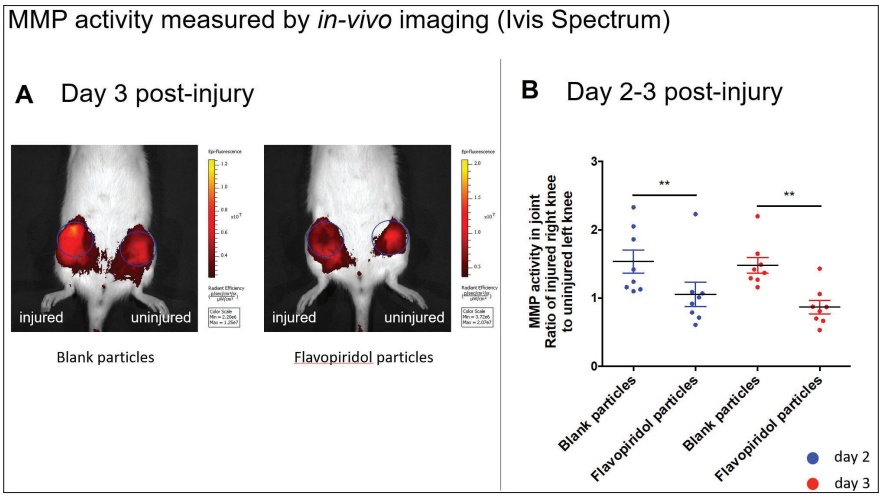

Figure 15: *In-vivo* testing of PLGA-encapsulated Flavopiridol in a rat model of PTOA. PTOA was induced in rats using our ACL-Rupture model. At the time of injury, 4mg of PLGA-Flavopiridol (or blank PLGA) microparticles were administered in 50 μl of saline via intra-articular injection. After 24 hours, and live-animal imaging was used to determine MMP activity in the injured and uninjured contralateral joints. A) Image of typical rat (n=8/group) at day 3 showing Region of Interest (ROI) in blue circle. B) quantification of MMP activity in the ROI in all rats at day 2 and day 3 post-ACL-rupture.

(g) **PLGA-encapsulated Flavopiridol in an ACL-rupture rat model of PTOA**

We tested the *in-vivo* performance of the PLGA-encapsulated Flavopiridol in our non-invasive ACL-Rupture rat model of post-traumatic osteoarthritis. The results in figure 15 (page 23), indicate that a single intra-articular injection of PLGA-Flavopiridol greatly reduces the MMP activity within the joint space after ACL-rupture injury. We are in the process of obtaining later time points for the MMP activity, and also to grade the severity of osteoarthritis in these joints. The data looks promising. In several animals we see a 'black hole' of MMP activity (figure 15A right panel) where the MMP activity in the injured joint is even lower than the basal amount in the contralateral uninjured joint.

Please note that although these experiments made use of the PLGA-Flavopiridol microparticles, the animal experiments were not paid for using CDMRP funds. This is the continuation of the study we showed as preliminary data in the original grant application, and was performed with the IACUC approval we already had in place. This distinction is important because we did not propose any rat experiments as part of this grant. I include the data because it made use of the PLGA-Flavopiridol microparticles, and because it provides sufficient replicates (n=8/group) to publish the study, whereas the preliminary data at the time we wrote this grant only had 2 animals per group.

(h) **Biodistribution of PLGA and of Flavopiridol in the synovial capsule**

The biodistribution of PLGA, and of Flavopiridol in the joint remain unknown. To assess the biodistribution of these components, we labeled them with fluorescent dies Sulfo-Cy5 to label Flavopiridol, and Sulfo-Cy7 to label PLGA. These fluorescent dies can be excited with far-red wavelengths and emit at near-IR wavelengths. As such, they can be used for *in-vivo* imaging to localize PLGA and Flavopiridol in the joint space over time, and also in histological sections after animals are euthanized. The structures of the dyes are given

in figure 16 (page 24). We next tested the loading efficiency of the fluorescently labeled flavopiridol in PLGA microparticles using a variety of techniques as compared to the fabrication process developed by Tesio at Phosphorex. These results are shown in figure 17 (page 24) and demonstrate that a high encapsulation efficiency is possible with fluorescently labeled Flavopiridol. Results on the biodistribution of the Flavopiridol and PLGA will be reported in the next progress report.

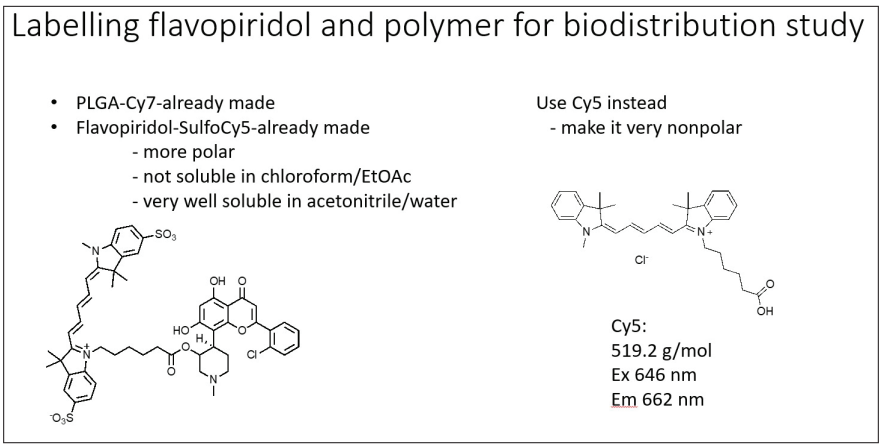

Figure 16: Biodistribution studies showing fluorescent dies used to label PLGA and Flavopiridol

Loading efficiency of Fluorescent Flavopiridol

samples	formulation method	% loading efficiency	mmol flavopiridol/mg particle	loading efficiency relative to company particles
flavopiridol particles	company	60	3.0E-05	1.00
flavopiridol particles	single emulsion	36	2.1E-05	0.71
flavo-cy5 particles	single emulsion	39	6.6E-06	0.22
flavo-cy5 particles	double emulsion condition 1	79	1.4E-05	0.48
flavo-cy5 particles	double emulsion condition 2	68	2.2E-05	0.75

Figure 17: Biodistribution studies showing fluorescent dies used to label PLGA and Flavopiridol

4. **CT Imaging and Image Analysis** (Collaborator Emeritus Professor Maury Hull, PhD)
Year 1 of this proposal mostly consisted of *in-vitro* work, while Year 2 will include a lot of large-animal imaging. Toward this, we obtained ovine cadaver legs to image on CT and MRI to establish a procedure for large-animal imaging, and to get experience training the personnel that will be recruited to perform and assist in the imaging. These are often students in the UC Davis School of Veterinary Medicine. Our imaging Co-investigator (Assistant Professor Derek Cissel, DVM, Ph.D.) left UC Davis for a different career opportunity in the Boston area. We were fortunate to establish a collaboration with Emeritus Professor Maury Hull, who has extensive experience with MRI and CT imaging and image analysis. Through his connections we had access to a state-of-the-art high resolution CT system with unprecedented resolution. Representative images

are shown in figure 18 (page 25).

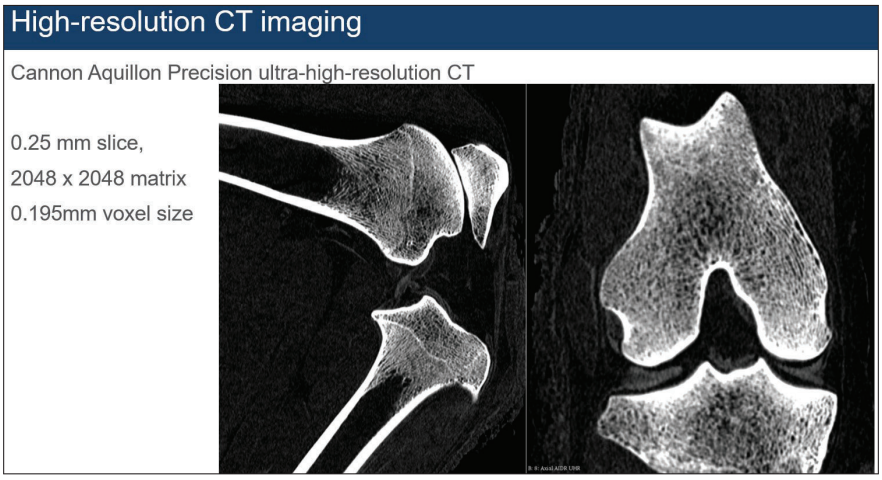

Figure 18: Ultra-high-Resolution CT images at various planes of view of a cadaver sheep stifle joint

The large datasets from high-resolution imaging requires development of new computer algorithms for data analysis. We purchased a limited license for MIMICS for image analysis. After using this software package we later determined this to be somewhat too limited, and the add-ons to make it a fully useful package were too expensive (upwards of $40k/year for the software that prefers to run on $20k computer hardware). A post-doctoral engineering fellow in Maury Hull's group (Anne Haudenschild, Ph.D.) suggested using the open-source software package Slicer. The Slicer app is primarily used in 3D printing to convert 3D objects into stacks of 2D-layers that are sent to the printer. With some adaptation, Slicer can be used to recreate 3D representations from 2D DICOM stacks such as those obtained in MRI and CT images. This Slicer software is free, and runs on much leaner hardware than Mimics. Anne Haudenschild programmed Slicer to accept the DICOM data from the high-resolution CT instrument, and established an AI/machine-learning based automated segmentation routine that greatly reduced the hands-on time for future analyses of these images. An example of such analysis is shown in figure 19 (page 26).

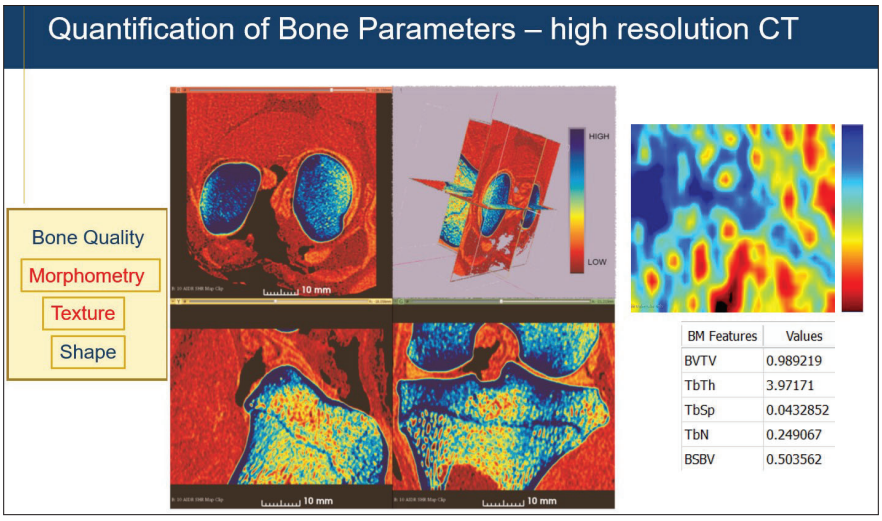

Figure 19: Image Analysis performed on high-resolution ovine stifle joint CT using Slicer software, showing a subset of parameters that can be quantified.

5. **Pharmacokinetics in Small and Large Animals** (Studies supported by internal funds)
   To be very clear, these small-animal and large-animal studies were *not* performed with PR171305 funds. The funds for the rodent studies were from my allotted pool of unrestricted departmental funds, and the equine studies were from an internal grant from the UC Davis Center for Equine Health (CEH) received by Co-Investigator Derek Cissell. Both studies used the Flavopiridol-PLGA extended-release formulations provided by Tesio, so they contribute to our progress toward commercializing the extended-release flavopiridol product for PTOA. This is why I am including the following data in this report.

   We wanted to understand the pharmacokinetics of flavopiridol and of extended-release formulations of flavopiridol in our microparticle and gel formulations *in-vivo* using both small and large animal models required by the FDA. For the small animal model we picked rats as the species, and *using UC Davis internal funds*, we performed PK analyses of two different amounts of free flavopiridol injected into the joints, as well as extended-release flavopiridol-PLGA in the microparticle and gel formulations. For all these studies, systemic exposure was measured in rats in blood collected from the saphenous veins at various time points out to 6 weeks.

   After intra-articular injection, free (unformulated) Flavopiridol persisted in the circulation with a half-life of approximately 70-90 minutes. The highest exposure was measured within 30 minutes after the drug was injected into the joint space, and that rapidly dropped to levels below the lower limit of quantification (50pg/ml) by 24 hours. The animals receiving 133ug Flavopiridol had plasma peaks of about 55ng/ml, whereas the animals receiving 13ug Flavopiridol had peak plasma levels of about 5.5ng/ml. These data are shown in figure figure 20 (page 27).

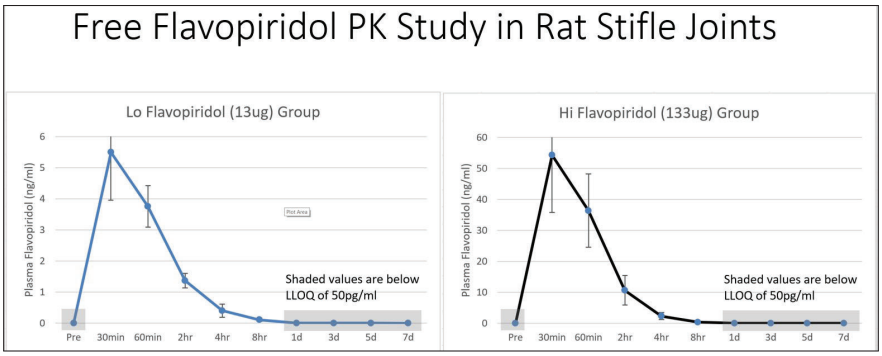

Figure 20: Pharmacokinetics analysis of free (unformulated) Flavopiridol after injection into rat stifle joints at two concentrations.

We next performed pharmacokinetics analysis of PLGA-encapsulated flavopiridol from both gel and microparticle formulations. The results suggest that an initial burst of flavopiridol is released into the plasma by both formulations within the first 24 hours, with a larger initial burst from the gel formulation. This is followed by a period of constant exposure just slightly above the lower limit of quantification of our assay, which is 50pg/ml in plasma. These *in-vivo* release kinetics satisfy our initial design criteria. The data are shown in figure 21 (page 27).

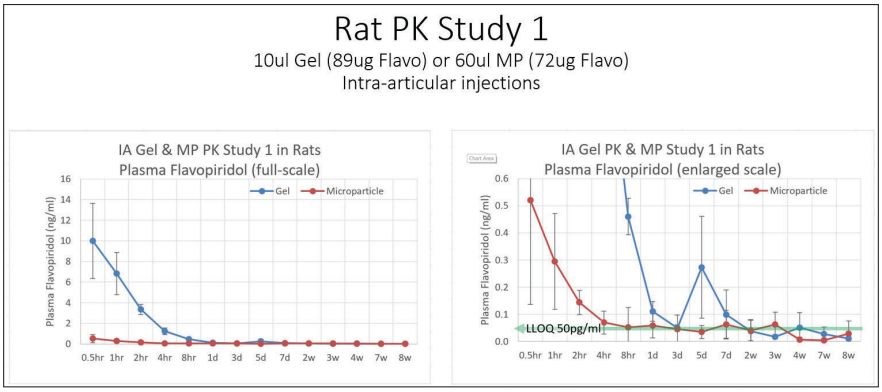

Figure 21: Pharmacokinetics analysis of PLGA-encapsulated Flavopiridol after injection into rat stifle joints. The Left panel shows full scale on the y-axis, the right panel shows a magnified y-axis scale to better visualize the lower concentrations observed at the later time points.

We also performed pharmacokinetics analysis of PLGA-encapsulated flavopiridol in microparticle formulations using an equine model, as described above. These data are now fully analyzed, and suggest that the flavopiridol remains detectable within the joint space for at least 3-4 weeks, meeting our design criteria. The gel formulation has somewhat more of a burst release than the microparticle formulation, as evidenced by higher plasma concentrations of Flavopiridol in the first 24 hours. This burst release could be an advantage because it provides immediate access to the anti-inflammatory properties of the flavopiridol. Or it could be a disadvantage because it exposes the tissues to a high drug concentration for a limited duration. Further experiments will be required to determine the desirability of the initial burst.

In Summary, in year 1 we made substantial progress in developing a sustained-release formulation of the Cdk9 inhibitor Flavopiridol for intra-articular injection. We made progress in laying the groundwork for

the sheep studies in year 2 with respect to imaging and image analysis, and additional studies into the biodistribution of the Flavopirdol and PLGA which will be useful for pre-IND meetings with the FDA.

▷ *Opportunities for training and professional development has the project provided?*

This is primarily a Therapeutic Development Grant, not a training grant. As UC Davis is an academic institution, we have several trainees working on this project. This includes PhD students in various programs, and post-doctoral fellows. A table of these personnel is given in table 1 (page 28). Regular training activities include participation in seminars across the various graduate groups and departments (Chemistry, Orthopaedic Surgery, School of Veterinary Medicine, Biomedical Engineering, Molecular Biology) as well as project-based group discussions and individual discussions with faculty mentors.

Table 1: Trainees

Gabriel Fraley	Haudenschild Group	PhD student in Biochemistry Molecular Biology
Dustin Leale	Haudenschild Group	PhD student in Integrated Pathobiology
Katherine Morucci	Cissell Group	PhD candidate in Anthropology (Finished)
Matthew Owen	Liu Group	PhD candidate in Chemistry
Marshall Van Zijll	Liu Group	Post-doctoral fellow in microparticle formulation (Finished)
Cody Chalker	Liu Group	Post-doctoral fellow in microparticle characterization
Anne Haudenschild	Hull Group	Post-doctoral fellow in biomechanics and imaging
Rapeepat Sangsuwan	Lewis Group	Post-doctoral fellow in microparticles

Table 2: Table of Trainees. Given the impact of COVID on hiring and room occupancy, no new hires were made during this reporting period. Trainees who have finished and moved on to different positions are indicated.

▷ *How were the results disseminated to communities of interest?*

Outreach was not specifically done for this project. However, advances in this project were posted on the various social media accounts maintained by the department. For example my home department of Orthopaedic Surgery maintains a webpage with my lab Twitter, Facebook, and LinkedIn feeds here (https://health.ucdavis.edu/orthopaedics/research/haudenschild.html) and here (https://ortho-research.faculty.ucdavis.edu/). Additional faculty have their own social media presence that reaches a broader audience than the scientific publications can.

▷ *What do you plan to do during the next reporting period to accomplish the goals?*

The plan is to continue to work on the major tasks and minor tasks as outlined in the Statement of Work document. Much of the personnel has been identified and brought on board. However, two Co-investigators left the University: Derek Cissel (MRI/CT imaging) and Chin-sang Chen (biostatistics). Maury Hull partially fills the role of MRI imaging, however a new collaborator will need to be identified to fill the role of Biostatistician. We plan to adjust the scope of the project to accommodate the realities of the COVID-19 situation, and the realities that 18-month time points are no longer considered the standard in OA research. In summary, we plan to eliminate the 18-month time point, and instead focus on the 6- and 12-month evaluations, with a majority of the savings applied toward the losses of productivity encountered during the mandated shutdowns of COVID-19.

Impact

This is the second year of the proposal, a majority of which was spent in COVID-19 lockdown. No major impacts have resulted yet.

▷ *What was the impact on the development of the principal discipline(s) of the project?*

Nothing to report.

▷ *What was the impact on other disciplines?*

Nothing to report.

▷ *What was the impact on technology transfer?*

We founded Tesio Pharmaceuticals, Inc. as a UC Davis-based startup company in February of 2017. The purpose of Tesio is to commercialize the research performed in this project. The company was initially 2 co-founders partaking in this grant (Dominik Haudenschild and Jasper Yik at UC Davis) and a 3rd co-founder who is an equine orthopaedic surgeon. In 2020, the company continues to grow substantially. We hired a CEO and a COO, who together accomplished a significant **milestone** by negotiating quite favorable terms of the intellectual property license with UC Davis.

We hired a team of consultants with experience in scale-up manufacturing of sustained-release injectables. We hired a team to advise us on IP strategy to build a strong patent portfolio. We hired another consultant with experience in setting up pre-IND/IND meetings with the FDA, and compliance with regulatory agencies. We hired a consultant with experience in toxicology studies, and a consultant with experience in pharmacokinetics, as we will still need to file a New Drug Application for Flavopiridol. We hired a consultant experienced in the GMP manufacture of small-molecule APIs. We hired two consultants experienced in sustained-release drug formulations, and a well-known key opinion leader (KOL) to lend credibility to our efforts. With this team and its expertise in place, Tesio has a solid corporate structure ready to license and commercialize the therapeutic being developed in this TTDA project.

Tesio initiated a pre-IND meeting Q4 of 2020, and the meeting is set to occur (virtually) in mid-April of 2021. Given that the regulatory requirements and clinical trials timeline are nearly established, we started to approach Venture Capital investors in Q1 2021 for Series A funding of $15M to $20M to get Tesio into Phase IIb human clinical trials. To date, our non-confidential pitch deck is with about 24 top-tier Venture Capital firms. We expect to 'nudge' them again once the results from our pre-IND meeting become available in mid-April.

▷ *What was the impact on society beyond science and technology?*

Nothing to Report.

Changes/Problems

▷ *Changes in approach and reasons for change*

In FY1 of this proposal there were no significant changes to the approach.

In FY2 of this proposal COVID-19 happened. We were forced to shut down all in-person research operations by the University of California, and slowly re-open. Even now, 12 months after the initial lock-down, we are not allowed to operate at full capacity. This caused significant delay. I made the decision not to terminate anybody's employment, in part because the duration of the lockdown was unknown at first, in part because of the difficulty in hiring the highly qualified personnel, and in part because I strongly felt that it was the right decision from a moral perspective. The result is that salaries continued to be paid at 100% while hands-on work was severely reduced. This negatively affected our productivity and timeline for the project. When possible, I re-assigned related tasks to people that could be performed virtually. For example, writing literature reviews, or analyzing single-cell RNASeq data using bio-informatics routines.

▷ *Actual or anticipated problems or delays and actions or plans to resolve them*

We were disappointed when Co-investigator Derek Cissel, DVM, PhD, and Co-investigator Chin-sang Li, PhD, decided to leave UC Davis to pursue their careers elsewhere. This required us to identify a replacement faculty for the imaging that was to be done by Derek. Emeritus Professor Maury Hull, PhD, is a partial replacement for Derek, but his expertise is with human patients and not sheep. We still need to identify an appropriately skilled veterinary collaborator with ovine imaging interests. I do not anticipate that this will be difficult, as UC Davis is the top-ranked veterinary school nationally and there is a strong and very advanced imaging core. To fill the biostatistics role, I have established a working relationship with Professor Bradley Pollock, Department Chair of Epidemiology at UC Davis Health. Brad, or someone in his group, will join our team as biostatistician when it is appropriate.

To recover from COVID-related delays, we plan to eliminate the 18-month time point for sheep OA evaluation. This task is both the longest duration, and the most costly task in the proposal. In addition, we plan to request a No-Cost-Extension to the project. This should allow us to finish most of the completed work. Finally, we continued development of the encapsulated flavopiridol drug product at Tesio (at Tesio's expense), and we expect to transfer some of this back to UC Davis.

▷ *Changes that had a significant impact on expenditures*

The cost of housing sheep increased dramatically since the time we submitted the grant budget. This project involves 120 sheep, housed up to 19 months, for a total of approximately 38,500 sheep-days. At the time we wrote the budget, the actual cost of housing sheep in an outdoor pen was $7.90/sheep/day. Anticipating some increases, I wrote the budget assuming $15/sheep/day, and included a total of about $580,000 for the housing of sheep. To our great surprise, for FY2020-21 (which starts in July 1 of 2020), the University increased the cost of housing sheep to $28.90/sheep/day, or almost twice what I budgeted for. I am negotiating with the University that they provide a 'grandfather' clause in their price increase for grants such as this. The University is also offering bridge-grants to accommodate situations such as ours, and I am applying to those bridge grants to help cover the gap. Depending on the outcomes of

these negotiations, we may have to scale back some of the MRI imaging at the intermediate time points to focus more on the primary endpoint outcomes rather than continuously imaging the progression of OA development in the sheep.

An update to the cost of sheep housing is that I have reached an agreement with the University that the housing for sheep in this project will be fixed at $15/day/sheep for the remainder of the study.

▷ *Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents*

There was delay in the approval of our IACUC protocol due to its complexity. We are working to address this. We split the IACUC protocol into two parts: PK analyses, and OA efficacy studies. The IACUC for PK analysis was approved early 2021, followed shortly by ACURO approval. We have initiated the PK studies in sheep with the first set of animals having received a dose at the end of March 2021. We learned from this, and are refining the techniques to be included in the OA efficacy studies IACUC proposal.

▷ *Significant changes in use or care of human subjects*

Not Applicable, there are no human subjects

▷ *Significant changes in use or care of vertebrate animals.*

Nothing *major* to report other than the delay in IACUC approval (mentioned above). We are making minor refinements to the techniques for arthrocentesis. For example, for future experiments we would like to include ultrasound guidance to precisely identify the location of synovial fluid in the joint space.

▷ *Significant changes in use of biohazards and/or select agents*

Nothing to report.

Products

▷ *Publications, conference papers, and presentations*

- Oral Presentation, Poly 273. Symposium "Polymer-Based Gene & Drug Delivery Systems." 257^{th} American Chemical Society National Meeting and Exhibition, Orlando, FL, 3/31-4/4, 2019. Matthew J. Owen, Jasper H.K. Yik, Congwang Ye, Hai Yu, Xi Chen, Dominik R. Haudenschild, Gang-yu Liu. Title: "Microfluidic synthesis of drug-loaded PLGA microparticles: A greener approach."
- Oral Presentation, Workshop of "Controlled Molecular Assembly." UC Davis, CA, 10/15, 2019. Matthew J. Owen, Jasper H.K. Yik, Congwang Ye, Hai Yu, Xi Chen, Dominik R. Haudenschild, Gang-yu Liu. Title: "Microfluidic synthesis of drug-loaded PLGA microparticles: A new and greener approach."

- Virtual Presentation Graduate Group in Integrative Pathobiology, UC Davis. Dustin M. Leale, Jamie White, Derek Cissel, Linan Li, Jasper Yik, Dominik R. Haudenschild. Title: "Reduction of Post-Traumatic Osteoarthritis by Inhibition of Primary Response Genes."
- Virtual Presentation at Arthritis Foundation 2020 Osteoarthritis Clinical Studies Forum (OACS). Dominik R. Haudenschild, Jasper HN Yik, Tomoaki Fukui, Jamal Lewis, Gang-yu Liu. Title: "Reducing PTOA Severity with Early Therapeutic Intervention."
- Virtual Presentation at Orthopaedic Research Society 2021 Annual Meeting. Yihan Li, Jasper HN Yik, Dustin M. Leale, J. Gabriel Fraley, Dominik R. Haudenschild. Title: "Cdk9 inhibition suppresses macrophage recruitment in ACL-rupture mouse PTOA model. "
- Virtual Presentation at UC Davis Graduate Group in Integrative Pathobiology. Dustin M. Leale, Matthew Settles, Keith Mitchell, Aimy Sebastian, Linan Li, Gabriela G. Loots, Jasper HN. Yik, and Dominik R. Haudenschild. Single cell RNA seq workflow for the analysis of whole murine knee joints.

▷ *Journal publications*

Since this is work toward a product for eventual commercialization, we decided against publishing results in real-time to allow time for appropriate patent filings. The COVID-19 shutdown prevented in-person bench science and forced remote working. During this time our team spent a considerable amount of (paid) time finalizing and submitting journal publications that were in various stages of completion. I include these efforts in this report, not because they were part of the original scope of the project, but because members of the team were being paid during this time.

- Manuscript: Fukui T, Yik JHN, Doyran B, Davis J, Haudenschild AK, Adamopoulos IE, Han L, and Haudenschild DR. Bromodomain-containing-protein-4 and Cyclin-Dependent-Kinase-9 Inhibitors Synergistically Reduce the Severity of Murine Post-Traumatic Osteoarthritis. Osteoarthritis and Cartilage, 29(1): 68-77.

▷ *Books or other non-periodical, one-time publications*

Nothing to Report.

▷ *Other publications, conference papers, and presentations*

Nothing to Report.

▷ *Website(s) or other Internet site(s)*

Occasional blog entries are published on my lab website's blog, for example the training we received on sheep gait analysis. `https://orthoresearch.faculty.ucdavis.edu/gait-analysis-for-sheep/`

▷ *Technologies or techniques*

Nothing to Report.

▷ *Inventions, patent applications, and/or licenses*

We are actively pursuing strong patent protection that will enable commercialization of the therapeutic being developed within this award and with our company Tesio Pharmaceuticals, Inc. Much of the University-based research is being patented by the University's Office of Technology Transfer. However, since Tesio is negotiating to license this IP, Tesio's legal team is also involved in the patent filings. The newer patent applications are being filed jointly with both UC Davis and Tesio Pharmaceuticals listed as inventors. There are three patent families that relate to this project:

- Use of CDK9 inhibitors to reduce cartilage degradation
  - US Patent 9,498,471
  - US Patent 10,172,844
  - US Patent 10,639,302
  - Additional US continuations pending
- Use of CDK9 and BRD4 inhibitors to inhibit inflammation
  - US Patent 10,300,073
  - EU Patent Notice of Allowance received 2021
  - Foreign patents pending in Canada, Japan, Australia, Hong Kong
  - Additional US and Foreign continuations pending
- Sustained Release Formulation for Local Delivery of Cdk9 Inhibitors
  - International Application No. PCT/US2019/028721 (jointly filed by UC Regents and Tesio Pharmaceuticals, Inc.

▷ *Other Products*

Nothing to Report.

Participants & Other Collaborating Organizations

▷ *What individuals have worked on the project?*

Name:	***Dominik R. Haudenschild, PhD***
Project role:	*Principal Investigator*
Research identifier	*ORCID ID https://orcid.org/0000-0001-9947-9864*
Nearest person month worked	*7*
Contribution to project	*Overall direction of project*
Funding support	*This grant, departmental funds, and NIH R01*

Name:	***Jasper H.N. Yik, PhD***
Project role	*Co-Investigator*
Research identifier	*https://orcid.org/0000-0001-7532-629X*
Nearest person month worked	*11*
Contribution to project	*Molecular biology of Ckd9 responses*
Funding support	*This grant, and NIH R01*

Name:	***Gang-yu Liu, PhD***
Project role	*Co-Investigator*
Research identifier	*https://orcid.org/0000-0003-3689-0685*
Nearest person month worked	*1*
Contribution to project	*Formulation and Characterization of microparticle*
Funding support	*This grant, department, NSF, and NIH*

Name:	***Jamal Lewis, PhD***
Project role	*Co-Investigator*
Research identifier	*https://orcid.org/0000-0002-9811-8538*
Nearest person month worked	*1.5*
Contribution to project	*Formulation and Characterization of microparticle*
Funding support	*This grant, department, and NIH*

Name:	***Ron K. June, PhD***
Project role	*Co-Investigator*
Research identifier	*https://orcid.org/0000-0003-0752-4109*
Nearest person month worked	*1*
Contribution to project	*Metabolomics quantification of Flavopiridol in body fluids*
Funding support	*This grant, department, and NIH*

Name:	***Rapeepat Sangsuwan, PhD***
Project role	*Post-doctoral Fellow*
Research identifier	*N/A*
Nearest person month worked	*6*
Contribution to project	*Microparticle fabrication and testing*
Funding support	*This grant, department, and NIH*

Name:	***Marshall Van Zijll, PhD***
Project role	*Post-doctoral Fellow*
Research identifier	*N/A*
Nearest person month worked	*6*
Contribution to project	*Microparticle fabrication and testing*
Funding support	*This grant, department, and NIH*

Name:	***Cody Chalker, PhD***
Project role	*Post-doctoral Fellow*
Research identifier	*N/A*
Nearest person month worked	*6*
Contribution to project	*Microparticle fabrication and testin*
Funding support	*This grant, department, and NIH*

Name:	***Anne K. Haudenschild, PhD***
Project role	*Post-doctoral Fellow*

Research identifier	*N/A*
Nearest person month worked	*6*
Contribution to project	*MicroCT Image acquisition and analysis*
Funding support	*This grant, department, and NIH*

Name:	***Matthew Owen***
Project role	*Graduate Student*
Research identifier	*N/A*
Nearest person month worked	*2*
Contribution to project	*Microparticle fabrication and characterization*
Funding support	*This grant, department, and NIH*

Name:	***Dustin Leale***
Project role	*Graduate Student*
Research identifier	*N/A*
Nearest person month worked	*8*
Contribution to project	*Molecular Characterization of Ckd9 responses*
Funding support	*This grant, department, and NIH*

▷ *Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?*

Nothing to Report.

▷ *What other organizations were involved as partners?*

Co-investigator Associate Professor Ronald K. June at Montana State University was involved, exactly as described in the original grant application, as a sub-contract site to perform the metabolomics quantification of Flavopiridol in synovial fluid and serum samples.

Tesio Pharmaceuticals Inc. is involved as a commercialization partner. Tesio has finalized the license agreement with UC Davis for the commercialization of relevant University-owned intellectual property. A joint patent has been filed listing both UC Regents and Tesio Pharmaceuticals as inventors. At the moment, Tesio is a privately held virtual company incorporated in Delaware as a C-corp, and with "headquarters" at Dominik Haudenschild's personal residence.

Special Reporting Requirements

▷ *Collaborative Awards*

Not Applicable.

▷ *Quad Charts*

Not Applicable.

Appendices

None