Intra-articular injection of an extended-release flavopiridol formulation represents a potential alternative to other intra-articular medications for treating equine joint disease

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OBJECTIVE
To establish the pharmacokinetics of the cyclin-dependent kinase-9 inhibitor flavopiridol in equine middle carpal joints, using an extended-release poly lactic-co-glycolic acid (PLGA) microparticle formulation.

ANIMALS
4 healthy horses without evidence of forelimb lameness.

METHODS
A 6-week longitudinal pharmacokinetic study was conducted in 2 phases (6 weeks each) in 4 healthy horses. The PLGA microparticles containing 122 μg flavopiridol in 3 mL saline were administered by intra-articular injection into 1 middle carpal joint, with empty PLGA microparticles injected into the contralateral joint as a control. Synovial fluid and plasma were collected at time points out to 6 weeks, and drug concentrations in synovial fluid and plasma were determined using validated protocols. Synovial fluid total protein and total nucleated cell count and differential, CBC, serum biochemistry, and lameness exams were performed at each of the time points.

RESULTS
Synovial fluid flavopiridol averaged 19 nM at week 1, gradually reduced to 1.4 nM by 4 weeks, and was generally below the detection limit at 5 and 6 weeks. There was no detectable flavopiridol in the plasma samples, and no adverse effects were observed at any time point.

CLINICAL RELEVANCE
Intra-articular injection of PLGA microparticle-encapsulated flavopiridol was well tolerated in horses, with detectable levels of flavopiridol in the synovial fluid out to 4 weeks with negligible systemic exposure. Flavopiridol is a cyclin-dependent kinase-9 inhibitor with potent anti-inflammatory and analgesic activity. The extended-release microparticle formulation promotes intra-articular retention of the drug and it may be an alternative to other intra-articular medications for treatment of equine joint disease.

Keywords: osteoarthritis, equine, flavopiridol, intra-articular, pharmacokinetics

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common features of OA, and therapeutic interventions typically include anti-inflammatory treatments such as administration of intra-articular (IA) medications. While a variety of IA therapies are available, including biologic products (autologous conditioned serum, platelet-rich plasma, and stem cells), polyacrylamide hydrogels, hyaluronic acid, and polysulfated glycosaminoglycans among others, injection of corticosteroids remains the most commonly employed IA treatment. \(^5\) \(^6\) \(^7\) However, the use of IA corticosteroids such as triamcinolone acetate and methylprednisolone acetate has been shown to have detrimental effects in addition to their potent anti-inflammatory activity. Intra-articular corticosteroid use in horses has been associated with the development of lamineitis, joint flares, and loss of articular cartilage volume among other negative consequences, particularly with repeated administration. \(^7\) Due to the adverse effects OA can have on the individual horse, and the overarching negative impact OA has on the equine industry as a whole, there is an ongoing clinical need to develop new therapeutic strategies aimed at the prevention and treatment of OA.

Recent research demonstrates the role of cyclin-dependent kinase-9 (CDK9) in the development of OA following joint injury. \(^8\) \(^9\) \(^10\) \(^11\) Cyclin-dependent kinase-9 is a rate-limiting step for the transcriptional elongation of primary response genes \(^12\) \(^13\) \(^14\) \(^15\) that include many inflammatory genes and mediators of pathways involved in OA pathogenesis. \(^8\) \(^9\) \(^10\) \(^16\) Pharmacological inhibition of CDK9 protected against the catabolic effects of proinflammatory cytokines in vitro \(^11\) and prevented mechanical injury-induced inflammatory cytokine production, apoptosis, and the subsequent degradation of cartilage matrix in bovine explant cultures. \(^10\) Pharmacological inhibition of CDK9 also reduced the severity of post-traumatic osteoarthritis (PTOA) in a mouse anterior cruciate ligament–rupture model of PTOA. \(^8\) \(^9\)

A number of small-molecule CDK9 inhibitors are being developed that either inhibit its kinase activity or target the protein for degradation. \(^7\) The CDK9 inhibitors are primarily being developed for use as chemotherapeutics to target CDK9 in certain malignancies, \(^18\) with the best-studied CDK9 inhibitor being flavopiridol (alvocidib). Flavopiridol is a semisynthetic small-molecule CDK9 inhibitor that has been evaluated in 67 clinical trials currently listed on clinicaltrials.gov. \(^18\) \(^19\) \(^20\) Most of the clinical trials using flavopiridol target CDK9 in the context of leukemia, which requires systemic IV administration of flavopiridol at high doses. These systemic studies determined that the maximum tolerated flavopiridol dose in humans and dogs is approximately 50 mg/m²/day. \(^21\) \(^22\) Studies in rats suggest that flavopiridol is metabolized by glucuronidation in the liver and excreted in the bile \(^23\) and has a plasma elimination half-life of about 2 hours. \(^22\)

In the diarthrodial joint, the synovial membrane provides a relatively impermeable barrier to the IA environment. This results in IA administration of medications being the preferred route of delivery to achieve therapeutic concentrations. Additionally, IA delivery of medications allows for local therapeutic concentrations with low systemic burdens. However, small-molecule drugs such as corticosteroids diffuse out of the IA environment rapidly, limiting their duration of activity. \(^24\) Newer generation IA small-molecule therapeutics are formulated using extended-release drug delivery strategies. Drug encapsulation in polylactic-co-glycolic acid (PLGA) is one of the most common approaches, as it serves to extend the duration of drug release as the PLGA is resorbed, and the PLGA particle size helps retain the drug within the joint space. \(^25\)

The aim of this study was to establish the tolerability and pharmacokinetics of a sustained-release PLGA formulation of flavopiridol administered by IA injection into equine middle carpal joints. We hypothesized that IA administration of a therapeutic concentration of flavopiridol would result in detectable levels of flavopiridol in the synovial fluid, with minimal systemic exposure as measured by plasma flavopiridol levels. Further, we hypothesized that IA flavopiridol would result in no difference in lameness, joint effusion, swelling, or synovial fluid parameters compared to administration of a placebo.

**Methods**

**Polylactic-co-glycolic acid microparticles**

The formulation, manufacture, and initial characterization of the PLGA microparticles was outsourced to Phosphorex Inc. At Phosphorex, the microparticles were filled as a suspension into glass vials under sterile conditions, then lyophilized and sealed. Each vial contained 122 μg flavopiridol in a total of 11.29 mg PLGA microparticles. The average particle size was 16.0 ± 8.4 μm. Sterility and endotoxin testing were performed by Cambrex using industry-standard tests. The total bioburden was < 12 CFU/sample item portion, and endotoxin levels were < 0.05 EU/mL, both clinically acceptable according to our understanding of FDA Guidance for Industry. In vitro release studies performed by Phosphorex indicated linear flavopiridol release out to 4 weeks, corresponding to approximately 4 μg/day. Preparation of blank PLGA microparticles was the same but without the incorporation of flavopiridol into the microparticles. At the time of IA administration, 3 mL sterile saline was added to each glass vial, which was then partially immersed in an ultrasonic cleaner bath (model FS20; Fisher Scientific) for 3 to 5 minutes to resuspend the particles fully.

**Primary cell isolation and culture**

Primary equine chondrocytes and synovioocytes were isolated ex-vivo from animals that were humanely euthanized for ailments unrelated to musculoskeletal disease (n = 3 horses). Following sedation with a 1.1 mg/kg IV injection of xylazine hydrochloride (AnaSed; Lloyd Inc), euthanasia was...
In vitro bioactivity and toxicity

Two in vitro bioactivity and toxicity assays were performed to estimate a safe dose with therapeutic efficacy. Primary equine chondrocytes or synoviocytes (n = 3 donors) were seeded in 96-well plates at 40% to 50% confluency by cell growth surface area. After overnight incubation, cells were treated with recombinant equine IL-1β to simulate an inflammatory environment (10 ng/ml; R&D Systems), in the presence of increasing concentration of flavopiridol (316, 100, 31.6, 10, 3.16, 1.0, or 0.3 nM or no flavopiridol) for 24 or 48 hours made in triplicate wells. After overnight incubation, cells were treated with serial dilutions of flavopiridol (316, 100, 31.6, 10, 3.16, 1.0, or 0.3 nM or no flavopiridol) for 24 or 48 hours made in triplicate wells. After overnight incubation, cells were treated with serial dilutions of flavopiridol (316, 100, 31.6, 10, 3.16, 1.0, or 0.3 nM or no flavopiridol) for 24 or 48 hours made as serial dilutions. The levels of mitochondrial LDH in the media were then measured using the CyQuan cytotoxicity kit (Thermo Fisher) according to the manufacturer’s protocol.

Equine inclusion criteria

Four horses were selected from the teaching herd maintained by the University of California-Davis Center for Equine Health, and all procedures were approved by the IACUC. All horses had physical and lameness examinations performed by 1 author (SAK) before enrollment in the study. Static lameness examination included palpation and manipulation of the forelimbs with special attention paid to the carpi. Dynamic lameness examination included observation of the horses at the trot in a straight line and in a circle in both directions. Following initial observation, upper and lower forelimb flexions were performed. All horses were deemed to be free of forelimb lameness arising from the carpi, and bilateral carpal radiographs were performed before inclusion in the study. Dorsopalmar, lateromedial, dorsolateral-palmaromedial, dorsomedial-palmarolateral, and flexed lateromedial projections were obtained with no appreciable pathologic changes observed. Follow-up carpal radiographs were obtained 4 and 14 months after completion of the study. Animals were housed on dry lots with water and food ad libitum for the duration of the study.

Intra-articular injection and arthrocentesis

Each horse was sedated with 3 to 5 mg detomidine hydrochloride (Zoetis) administered IV and appropriately restrained. The dorsal aspect of each carpus underwent aseptic preparation centered over the middle carpal joint using betadine and isopropyl alcohol. Arthrocentesis was performed using a 21-gauge needle, and approximately 3 mL of synovial fluid was collected for cytologic analysis. Without removing the needle, either 3.0 mL of saline containing PLGA-flavopiridol microparticles or 3.0 mL of saline containing blank-PLGA microparticles was injected into the joint. All injections and all subsequent synovial fluid collection analyses were performed by 1 author (SAK) who was blinded to which joint (left or right) received the flavopiridol or blank microparticles. For phase 1 of the study, bilateral middle carpal joint arthrocentesis was performed at weeks 0, 2, 4, and 6. For phase 2 of the study, bilateral middle carpal joint arthrocentesis was performed at weeks 0, 2, 4, and 6.
performed on weeks 0, 1, 3, and 5. The joint receiving flavopiridol microparticles in phase 1 received blank microparticles in phase 2, and vice versa, and there was a 5-week washout period between phases 1 and 2.

**Plasma pharmacokinetics**

Plasma was collected weekly for both phases 1 and 2. For full plasma pharmacokinetics analysis, in phase 2, we collected plasma at 30-minute, 1-hour, 2-hour, 4-hour, 8-hour, 24-hour, 2-day, 3-day, 4-day, and 5-day time points.

**Lameness grading**

At each time point and before arthrocentesis, each horse was observed, and video was recorded while walking and trotting in a straight line. Twelve months after the completion of data collection, the videos were randomized and independently evaluated by 3 equine veterinarians (SAK, MPN, and MDH) experienced in lameness evaluation. Lameness was graded using the 5-point scale described by the American Association of Equine Practitioners. In addition, the range of motion was assessed weekly, and carpi were examined for signs of periarticular swelling, effusion, or other signs of discomfort upon palpation or manipulation.

**Flavopiridol quantification in plasma and synovial fluid**

Flavopiridol was quantified in dipotassium-EDTA plasma and synovial fluid using LC-MS/MS with validated methods on a Waters Acquity I-Class UPLC and Xevo TQ-S Mass Spectrometer IVD system (Waters). The lower limit of quantification in plasma and synovial fluid was 500 pg/mL (1.25 nM) and 50 pg/mL (0.125 nM), respectively, and both assays were linear to 1,000 ng/mL. For comparison, flavopiridol is typically active at 10- to 300-nM concentrations. Additionally, CBC and serum biochemistry as well as routine synovial fluid analysis including total protein, total nucleated cell count (TNCC), and TNCC differential were performed at each time point.

**Statistical analysis and blinding**

A Huber robust outlier test was performed on all outcome measurements. To determine which experimental factors had a significant effect on pharmacokinetic properties, multivariable linear regression modeling was performed using multiple independent variables (drug formulation, postinjection time point, animal, experimental phase, and side of injected leg) against each single dependent outcome measurement. All data is reported as mean ± SD. To evaluate flavopiridol concentration in the synovial fluid and synovial fluid cytology over time, a matched-pair t test with Tukey post hoc analysis was performed at each time point with significant differences (P < .05). All statistics were performed using JMP v15 (SAS).

**Results**

**In vitro bioactivity and cytotoxicity**

Bioactivity assay indicated that flavopiridol inhibited IL-6 expression at an EC50 of approximately 20 and 30 nM in IL-1β-treated chondrocytes and synoviocytes, respectively. Moreover, at concentrations...
of 100 nM or above, flavopiridol completely suppressed IL-6 expression (Figure 1). In contrast, cytotoxicity was not detected across all samples, even at 300 nM of flavopiridol, which is 4 orders of magnitude greater than the EC50. No significant differences were detected in cytotoxicity or bioactivity between chondrocytes and synoviocytes.

**Equine safety and tolerability**

Four mature horses aged 8 to 11 years with weights ranging from 500 to 700 kg were enrolled in the study. There were 3 geldings and 1 mare, and breeds included 3 Warmbloods and 1 Lusitano. A target therapeutic concentration of 20 to 100 nM (0.8 to 40 ng/mL) flavopiridol was estimated from the in vitro bioactivity (hydroxyethyl cellulose) and toxicity (chondrocyte) analyses (Figure 1). Based on the linear 4-week in vitro release profile (~4 μg/day flavopiridol release into an estimated 5 mL synovial fluid) from our flavopiridol-PLGA microparticle formulation, we decided on an initial dose of 11 mg microparticles containing 120 μg (305 nm) of flavopiridol for the study.

All 9 injections of PLGA-flavopiridol microparticles and all 8 injections of PLGA-blank microparticles were well tolerated by the 4 horses enrolled in the study. There were no adverse effects noted at any time point. We did not observe a decreased range of motion, swelling, effusion, or other signs of discomfort upon palpation or manipulation of the carpi at any time point. Mild and transient lameness was observed at some time points but not significantly correlated with either the flavopiridol-PLGA or the blank-PLGA injections.

**Synovial fluid flavopiridol concentrations**

Flavopiridol remained detectable in the synovial fluid for at least 4 weeks. A Huber outlier test identified 1 outlier in the flavopiridol concentration and the total nucleated cell measurements that were excluded from further analysis. Multivariable linear regression modeling indicated that the study phase did not have a significant effect (P = .9912), so data from phases 1 and 2 were combined into 1 data set for further analysis. Peak synovial flavopiridol concentrations of approximately 19 nM were observed after 1 week, with gradually reducing amounts detected in weeks 2 to 4 (Figure 2). By 5 weeks, the synovial fluid concentrations were below the lower limit of detection. A full pharmacokinetic analysis of plasma flavopiridol (maximum and minimum concentration, time to maximum concentration, and area under the curve, etc) was not possible because flavopiridol was not detected or fell below the lower limit of quantification of 500 pg/mL at all time points.

**Synovial fluid cytology**

Clinical analysis of the synovial fluid indicated a statistically significant difference in TNCC between treated and untreated joints at weeks 1 and 5, with flavopiridol-PLGA–treated joints having a lower TNCC (Figure 3). There were no statistically significant differences in total protein, nucleated cell differential, or RBC concentration between joints receiving flavopiridol-PLGA or blank-PLGA microparticles. (Figure 4). At baseline, TNCC averaged 60 cells/μL. We observed statistically significant increases (P < .04) in mean TNCC at all subsequent time points, ranging from approximately 100 to 325 cells/μL. However, while increases in TNCC were observed, values remained within the normal reference range (< 500 cells/μL) for the duration of the study.

**Radiographic findings**

There was no radiographic evidence of joint pathology before the start of the study, or at the follow-up time points of 4 and 14 months after study completion.
The aim of this study was to establish tolerability and pharmacokinetics of IA injection of an extended-release PLGA formulation of flavopiridol in equine middle carpal joints. The main findings of this study are, first, that IA administration of an extended-release flavopiridol-PLGA microparticle formulation is well-tolerated in healthy horses and, second, that the formulation produces detectable levels of flavopiridol in the synovial fluid for approximately 4 weeks postadministration with negligible systemic exposure. Additionally, no adverse effects were observed during the entire study period. We did observe a slight, statistically significant increase in the synovial fluid TNCC, which remained within the normal range (< 500 cells/μL) for all joints and at all time points. These findings are comparable to

Discussion

Figure 4—Synovial fluid clinical analysis (A through F) demonstrates all values are in the normal range at all time points, with no negative consequences upon administration of blank-poly lactic-co-glycolic acid (PLGA) or flavopiridol-PLGA.
a previous study evaluating the effects of repeated arthrocentesis of the middle carpal joint in horses over an 8-week period. In that study, no IA medications were administered and TNCC remained within the normal range at all sampling time points for the duration of the study. This would suggest that the increased cellularity observed in the current study was a result of repeated arthrocentesis, and less likely as a reaction to the PLGA microparticles, although it has been established that PLGA itself can be a mild inflammatory stimulus locally. The increased cellularity was less pronounced for joints injected with flavopiridol-PLGA microparticles than blank-PLGA microparticles, which was anticipated given the anti-inflammatory activity of flavopiridol.

Corticosteroids are among the most common IA injections given to manage painful arthritic joints. Relatively high doses are typical, as soluble formulations of small-molecule drugs such as corticosteroids are rapidly lost from the joint into the general circulation, with serum peak levels typically within the first 12 hours and complete clearance within the first week. Because of the relatively high doses used, systemic side effects secondary to IA corticosteroid administration occur frequently, and these side effects have been thoroughly documented. Briefly, endocrine side effects include reversible suppression of adrenocorticotropic hormone and serum cortisol levels through the hypothalamic-pituitary-adrenal axis. Metabolic effects include transiently increased blood glucose levels (especially in diabetics), decreases in markers of bone turnover and formation but not bone resorption, and growth suppression in children. Hematological effects include reductions in blood mononuclear cells and increases in polymorphonuclear leukocytes. Additional local effects of IA corticosteroid injections can include loss of joint proteoglycans and changes to the mechanical integrity of cartilage, chondrocyte cell death (especially in combination with lidocaine), and osteoporosis with long-term or repeated use. Because these systemic side effects of soluble corticosteroids can be quite severe, there is an ongoing effort to reduce the systemic exposure through formulations that remain in the joint capsule upon injection. Common approaches include microcrystalline suspensions that dissolve slowly and extended-release formulations of steroids in bioresorbable polymers such as PLGA. In these formulations, local corticosteroid concentrations remain therapeutically effective, with lower total doses, reduced systemic exposure, and correspondingly fewer systemic side effects. However, a significant loss of joint proteoglycan content was still observed in dogs even with a locally administered extended-release formulation of triamcinolone-acetonide. CDK9 inhibitors such as flavopiridol can prevent the transcriptional activation of inflammatory genes and, as such, act through a different molecular mechanism to reduce inflammation than corticosteroids.

The CDK9 inhibitor flavopiridol was the first CDK inhibitor primarily developed as a chemotherapeutic agent against certain leukemias. As such, a high systemic dose of flavopiridol (at approximately 1- to 2-μM plasma concentrations) is required to show efficacy in treating leukemia, by inducing cell cycle arrest and apoptosis of cancer cells in human patients. In contrast, our data showed that a markedly reduced flavopiridol concentration (order of magnitude lower) is sufficient to effectively inhibit the expression of inflammatory genes, without significant cytotoxicity (Figure 2). In addition, local delivery of flavopiridol into the joint space will only require a fraction of the total body dose and further reduce systemic drug exposure and side effects. Our data show that a peak flavopiridol synovial concentration of 19 mM was achieved. While this is close to the target therapeutic concentration of 20 to 100 nM, the dose may need to be adjusted and further preclinical development will need to include a dose-escalation study.

Limitations of this study are that it was performed in only 4 horses, it was not a terminal study, and no tissue biopsies were taken; thus, we could not histologically assess the joint tissues for signs of local toxicity due to prolonged exposure to flavopiridol. However, we would not expect significant local toxicity at the low concentrations of flavopiridol, and there is evidence that flavopiridol can even be anti-apoptotic in bovine cartilage explants subjected to mechanical injury. Additionally, no evidence of detrimental effects on the joints was observed clinically or radiographically. Lameness was subjectively graded, and future studies may include objective measures.

An additional limitation is that to obtain weekly data points of synovial fluid flavopiridol concentrations, a 2-phase study design was deemed necessary by the IACUC to avoid weekly repeated arthrocentesis. Phase 1 provided data at the 2-, 4-, and 6-week time points, while phase 2 provided data at the 1-, 3-, and 5-week time points. To avoid possible confounding effects from repeated flavopiridol injections in the same joint, the opposite joint received the flavopiridol-PLGA microparticles in phase 2.

A further limitation is that we were unable to detect systemic plasma flavopiridol given the 500 pg/mL lower limit of quantification of our HPLC-MS method. Thus, a full pharmacokinetic analysis was not possible in this study. The advantage of a locally administered extended-release formulation is that it achieves locally therapeutic concentrations of drug with low systemic burden. We believe that an IA dose high enough to produce detectable levels of plasma flavopiridol would result in local toxicity within the joint. Future studies might include a 2 and 5 times escalation in dose, but even at these higher doses, we do not expect to detect plasma flavopiridol in 500- to 700-kg animals.

In summary, we demonstrate that an IA injection of extended-release flavopiridol-PLGA is well tolerated in healthy equine middle carpal joints. The pharmacokinetics show flavopiridol persists in synovial fluid for at least 4 weeks with no detectable systemic exposure. Future studies are needed to test the beneficial effects of flavopiridol in joint disease and reinforce its potential as an IA medication in horses.
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Disclosures

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