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“Bromodomain-containing-protein-4 and Cyclin-Dependent-Kinase-9 Inhibitors Interact Synergistically *in-vitro* and Combined Treatment Reduces Post-Traumatic Osteoarthritis Severity in Mice”

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Abstract

Objective.—Joint injury rapidly induces expression of primary response genes (PRGs), which activate a cascade of secondary genes that destroy joint tissues and initiate post-traumatic osteoarthritis (PTOA). Bromodomain-containing-protein-4 (Brd4) and cyclin-dependent-kinase-9 (CDK9) cooperatively control the rate-limiting step of PRG transactivation, including pro-inflammatory genes. This study investigated whether Brd4 and CDK9 inhibitors suppress inflammation and prevent PTOA development *in-vitro* and in a mouse PTOA model.

Methods.—The effects of Brd4 and CDK9 inhibitors (JQ1 and Flavopiridol) on PRG and associated secondary damage were rigorously tested in different settings. Short-term effects of

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Author Contributions

All authors contributed substantially to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Competing Interest Statement

No commercial funding was received for the work. The University of California has patents and pending patents on some of the findings, with Drs. Haudenschild and Yik listed as inventors. Drs. Haudenschild and Yik are co-founders of Tesio Pharmaceuticals, Inc, which is in the process of licensing related intellectual property from The University of California.

inflammatory stimuli (IL-1 β , IL-6, TNF) on human chondrocyte PRG expression were assessed by RT-PCR and microarray after 5-hours. We quantified glycosaminoglycan release from IL-1 β -treated bovine cartilage explants after 3-6 days, and osteoarthritic changes in mice after ACL-rupture using RT-PCR (2-24hrs), *in-vivo* imaging of MMP activity (24hrs), AFM-nanoindentation (3-7days), and histology (3days-4wks).

Results.—Flavopiridol and JQ1 inhibitors act synergistically, and a combination of both almost completely prevented the activation of most IL-1 β -induced PRGs *in-vitro* by microarray analysis, and prevented IL-1 β -induced glycosaminoglycan release from cartilage explants. . Mice given the drug combination showed reduced IL-1 β and IL-6 expression, less *in-vivo* MMP activity, and lower synovitis (1.5 vs. 4.9) and OARSI scores (2.8 vs. 6.0) than untreated mice with ACL-rupture.

Conclusions.—JQ1 and Flavopiridol work synergistically to reduce injury response after joint trauma, suggesting that targeting Brd4 and/or CDK9 could be a viable strategy for PTOA prevention and treatment of early OA.

Introduction

Osteoarthritis (OA) is a slowly progressing degenerative joint disease and the most prevalent form of arthritis, currently affecting about 27 million people in the U.S.[1] and 72 million by 2030[2, 3]. Despite decades of research on OA pathology, there is still no cure. Clinical therapies are palliative and do not address the underlying disease, leaving the majority of patients unsatisfied[4, 5]. OA risk factors include a previous joint injury,[6] which are responsible for ~12% of symptomatic knee OA [7, 8]. About 50% of patients with an anterior cruciate ligament (ACL) or meniscal injury will develop symptomatic OA within 10-20 years[9], representing a 10-fold increased risk[10, 11]. Pro-inflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF), and IL-6, are upregulated in joints after trauma[12–15]. Inflammation is a central cellular injury response in perpetuating catabolic degradation of cartilage and impairing the ability to repair[16, 17]. Increasing evidence points to the roles of injury-induced inflammation and secondary damage in potentiating post-traumatic OA (PTOA). Hence, therapeutic intervention to suppress inflammation following joint trauma may prevent the initiation and progression of PTOA.

Inflammation in PTOA is initiated by various stimuli, including pro-inflammatory cytokines (IL-1 β , IL-6, TNF, etc.), and each activates its respective cell surface receptors and intracellular signaling mediators.[18] Recent studies revealed that these diverse inflammatory signaling pathways converge on a common rate-limiting step during the transactivation of primary response genes (PRGs)[19, 20]. The rate-limiting step of PRG activation is controlled by cyclin-dependent kinase-9 (CDK9) and its recruitment factor bromodomain protein 4 (Brd4). Transcription of PRGs is normally inactive because RNA polymerase II (Pol II) is paused after transcription initiation. Upon signal stimulation, Brd4 recruits CDK9 to the paused Pol II, where it phosphorylates the Pol II C-terminal domain to induce a conformational change that allows transcriptional activation of full-length mRNAs. [19, 20] Given CDK9's central role in PRG transactivation, it is an attractive therapeutic target for effectively blocking the manifestation of the inflammatory cascades. [21, 22]

We previously demonstrated that the CDK9 inhibitor Flavopiridol (Flavo), which inhibits CDK9 kinase activity, suppressed *in-vitro* inflammatory responses in chondrocytes and the associated catabolic changes in cartilage tissue[21, 22]. A recently developed small molecule, JQ1, inhibits Brd4 to block CDK9's recruitment to gene promoters and has anti-inflammatory effects in mice[23]. Based on these studies, we postulate that combining Flavopiridol and JQ1, to block both CDK9's kinase activity and promoter recruitment, may produce additional anti-inflammatory benefits or effects. We hypothesize that treatment with combined CDK9/Brd4 inhibitors can effectively prevent injury-induced inflammation and progression of PTOA. In this study, we tested our hypothesis *in-vitro* using chondrocyte culture and cartilage explants, and *in-vivo* using an ACL rupture PTOA mouse model.

Materials and Methods

Chondrocyte isolation and expansion

Primary articular chondrocytes were isolated via enzymatic digestion from intact articular cartilage of healthy patients (n=5) receiving ACL reconstruction (ages: 18-45 years) as described previously,[24] using Institutional Review Board-approved protocol. Chondrocytes were isolated and cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). [25] Chondrocytes were used within three passages. All *in-vitro* experiments were repeated with chondrocytes from 3 to 5 donors plated at a cell density of 10,000 cells/cm² and allowed to reach 80% confluency prior to experiments, typically 1-2 days after plating.

Treatment of human chondrocytes with pro-inflammatory cytokines

Chondrocytes were treated with IL-1 β (10ng/ml), TNF- α (10ng/ml), or IL-6 (100ng/ml) plus IL-6 receptor (60ng/ml), in the presence or absence of Flavopiridol (Santa Cruz Biotechnology) and JQ1 (Cayman Chemical) at various dosages. A 5-hour timepoint was chosen based on our experience with peak PRG activation. Cells were washed with PBS, and RNA was extracted for qRT-PCR and microarray analyses.[21, 22]

Optimization of drug dosage

To determine the optimal drug dosage to suppress IL-1 β -induced inflammation, a statistical design of experiment (DOE) was created based on response surface methodology. Human chondrocytes from three donors were seeded in triplicate in 12-well plates and allowed to reach ~80% confluence. All groups were treated with 10ng/mL of IL-1 β to induce iNOS gene expression, and drugs were added alone or in combination following a Box-Behnken experimental design matrix (Fig 1A). Statistical modeling was performed as described by Derringer and Suich[26] to determine optimal dosing combinations based on complete repression of IL-1 β -induced iNOS gene expression, using in JMPv12 (SAS Institute, Cary, NC).

Microarray analysis

Microarray analysis was performed to examine the global effects of Brd4/Cdk9 inhibitors on PRG activation. Primary chondrocytes (n=2 donors) were treated with 10ng/ml IL-1 β with or without Brd4/CDK9 inhibitors for 5-hrs in 5 experimental groups: 1) IL-1 β only; 2)

IL-1 β with 1000nM JQ1; 3) IL-1 β with 250nM Flavopiridol; 4) IL-1 β with 250nM JQ1 and 60nM Flavopiridol; 5) untreated control. Total RNA was isolated as described below. RNA Integrity Number was >8 for all samples as determined on a Bioanalyzer (Agilent, Santa Clara, CA). Global mRNA expression in each sample group (n=2/group) was analyzed individually by Affymetrix GeneChip® Mouse Gene 1.0ST Array (Affymetrix, Santa Clara, CA). GeneSpring (Agilent Technologies, Santa Clara, CA) software was used to identify 873 genes that were induced >1.5 fold by IL-1 β treatment compared to untreated control. Differentially expressed genes were analyzed by hierarchical clustering using Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and visualized graphically using TreeView (<http://jtreeview.sourceforge.net/>). Raw microarray data are submitted to a public repository (<https://www.ncbi.nlm.nih.gov/geo/>).

Cartilage explants

Since inflammatory cytokines induce cartilage catabolism, we next determined the effects of Brd4/CDK9 inhibitors on IL-1 β -treated bovine calf cartilage explants. Full-thickness 6mm-diameter cartilage explants were harvested from femoral weight-bearing regions and trimmed to 2mm thickness while preserving the articular surface (Figure 2A). Explants were cultured for three days in DMEM with 10% FBS for recovery, then treated with IL-1 β (10ng/ml) and drugs for an additional six days to allow sufficient accumulation of breakdown products. Six joints were used, one explant from each joint was randomly assigned to each group. There was no significant difference in the cartilage weights among the groups. Media and drugs were replenished at day 3.

Glycosaminoglycan release assay

At day 3 and 6 of the culture of bovine cartilage, the culture media was collected, and glycosaminoglycan (GAG) was quantified using the dimethyl-methylene blue (DMMB) colorimetric assay with chondroitin sulfate as the standard [22]. The total amount of GAG released into the medium was normalized to the wet weight of each explant (n=6/group).

ACL rupture PTOA mouse model

A total of 142 adult male BALB/cByJ mice (12-week-old at time of injury) were obtained from Jackson Laboratory (Bar Harbor, Maine). All animals were maintained and used in accordance with NIH guidelines and approved by UC Davis Institutional Animal Care and Use Committee. In 121 animals, the right knee was injured by a single mechanical compression, and animals housed after injury as previously described[27]. This injury causes a transient anterior subluxation of the tibia to produce ACL rupture and consistently leads to PTOA within four weeks.[27] The contralateral knees were used as uninjured controls. Mice were isoflurane-anesthetized during injury, and a single dose of buprenorphine (0.05mg/kg) was administered immediately after injury. Euthanasia was by CO₂ asphyxiation.

Treatment of mice after knee injury

After injury, mice received systemic drug doses administered by intraperitoneal injections of the following: vehicle only (Ctrl), 50mg/kg of JQ1 (Hi J), 7.5mg/kg of Flavopiridol (Hi F),

or 17mg/kg of JQ1 and 2.5mg/kg of Flavopiridol (Lo Comb). Injections were given immediately after injury then 3x/week for up to 4 weeks.

Harvesting of whole knee joints for RNA isolation

At 2, 4, 8, and 24 hours post-injury, mice were sacrificed (n=4/group), and both knees dissected. Skin and muscle around knee joints were removed, and the whole knee joints were taken by cutting at the distal femoral growth plate and the proximal tibial growth plate. The tissues include articular cartilage, subchondral bone of femur and tibia, patella, meniscus, ligaments, synovia, and capsule. The collected tissues were frozen in liquid nitrogen and pulverized with a pestle and mortar under liquid nitrogen.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To quantify the effect of Brd4/Cdk9 inhibitors on injury-induced gene expression, total RNA was extracted from human chondrocytes or pulverized mouse knee tissues using miRNeasy Mini Kits (Qiagen Valencia, CA) and reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen). 2 μ l of cDNA was used for qRT-PCR, performed in triplicate using a 7900HT RT-PCR system (Applied Biosystems) with gene-specific TaqMan probes (Applied Biosystems, see Supplementary Table 1). Results were normalized to 18S ribosomal RNA. Fold change in expression relative to untreated controls was calculated using the 2^{-Ct} method.

In-vivo imaging of MMP activity

To image MMP activity within the joints, we used MMPsense750FAST fluorometric probes (Perkin-Elmer, Boston MA). Mice (n=4/group/timepoint) received injections of 2nmol MMPsense750 via the orbital sinus 0 or 24 hours, and were imaged 24 and 48 hours post-injury, respectively[28]. Briefly, mice under 3% isoflurane anesthesia were placed in an IVIS Spectrum imaging system (PerkinElmer), and the fluorescent signal of MMPsense750 was monitored using excitation/emission wavelengths of 745/800nm. Circular regions of interest (ROI) encasing the knee joints were created, and the average radiant efficiency [p/s/cm²/sr]/ [μ W/cm²] in the ROI was calculated as a measure of fluorescent signal intensity using Living Image software 4.2 (Perkin-Elmer).

AFM-nanoindentation of cartilage.

To quantify the effective indentation modulus of murine knee cartilage, Atomic Force Microscopy (AFM)-nanoindentation was performed on medial and lateral condyle cartilage surfaces at 3- and 7-days post-injury (n=6/group/timepoint), as previously described.[29] Briefly, bilateral distal femurs were dissected free of tendon and ligament tissues and glued onto AFM sample disks using cyanoacrylate adhesive gel (Loctite 409, Henkel Corp., Rocky Hill, CT). AFM-nanoindentation was performed on femoral condyle cartilage surfaces using a borosilicate colloidal spherical tip ($R\approx 5\mu\text{m}$, nominal $k\approx 7.4\text{N/m}$, AIO-TL, cantilever C, NanoAndMore, Lady's Island, SC) and a Dimension Icon AFM (BrukerNano, Santa Barbara, CA) in PBS with protease inhibitors (Pierce 88266, Rockford, IL). For each condyle, at least 10-15 different indentation locations were tested on the load bearing regions at 10 $\mu\text{m/s}$ AFM z-piezo displacement rate (approximate indentation rate) to account

for spatial heterogeneity. The effective indentation modulus, E_{ind} , was calculated by fitting the entire loading portion of each $F-D$ curve to the Hertz model, $F = 4E_{ind}R^{1/2}D^{3/2} / [3(1 - \nu^2)]$, where R is the tip radius, and ν is the Poisson's ratio (≈ 0.1 for cartilage [30]).

Histological analysis

At 3-, 7-, 14-, or 28-days, mice were euthanized (n=6/group, except n=5 for Injured group at 4wks, and n=7 for Injured+Drug group at 2wks), both knees dissected, fixed in 4% paraformaldehyde for 24h, and stored in 70% ethanol. Samples were demineralized in 10% formic acid with agitation for 48 hours, rinsed in PBS, and paraffin-embedded. Sagittal sections of 6 μ m were stained with hematoxylin and eosin and graded for synovitis,[31] or stained with Safranin-O and Fast-Green and scored for OA using the OARSI scoring system as the primary outcome[32]. OA scores from 3 blinded observers were the average of summed scores from the medial tibial plateaus and medial femoral condyles. Osteophytes were scored semi-quantitatively in 4-week sagittal sections adjacent to the anterior femur, posterior tibia, and anterior meniscus. A zero score indicates no osteophytes, the number (1, 2, 3) indicates how many of the 3 areas have osteophytes.[33]

Statistical analysis

Animal number calculations (n=6/group) for the primary outcome were based on a three-sample mean power analysis (power=0.8, p<0.05, standard deviation=0.3) using our previous histological OA score analysis [27] and a prediction that drug treatment would reduce OA grade by 50% (0.3). For secondary outcomes, qRT-PCR analyses include n=4/group/timepoint, n=8/group/timepoint for MMPsense imaging, n=6/group/timepoint for AFM measurements, and n=2 donors/group for microarray analysis. Mice were randomly assigned to experimental groups, assigned a number, and analyses were performed on numbered samples by blinded investigators. Statistical analysis of groups was performed in GraphPad PrismTM (MDF Software, Inc.), graphically testing for normal distribution, and using one-way ANOVA with Tukey's *post-hoc* analysis. Data were presented as mean \pm standard deviation. For all figures, differences were considered significant with p<0.05.

Results

Synergy between JQ1 and Flavopiridol in suppressing transactivation of the primary inflammatory response gene iNOS

To investigate the potential interactions between Brd4 (JQ1) and CDK9 (Flavopiridol) inhibitors on inducible gene expression, iNOS was chosen as a gene with a low baseline expression. Chondrocytes were stimulated with IL-1 β and the induced mRNA expression level set to 100% for comparisons. Experimental groups were given a wide range of JQ1 and/or Flavopiridol according to a Design of Experiment statistical approach (Fig1A). The statistical modeling predicted an inhibitor concentration contour plot at which iNOS induction is 100% suppressed by the combined inhibitors (Fig1B). The statistical model was significant (all p-values <0.0001) and adequate as determined by the lack-of-fit test (p<0.0001). When used alone, 1200nM of JQ1 or 250nM of Flavopiridol achieved 100% suppression. Optimizing the combined drug dosage resulted in 250nM of JQ1 plus 60nM of Flavopiridol for 100% suppression (Fig1B), representing a substantial reduction in the

individual drug dosages. The observation that the contour plot is a curve indicated there was a synergistic interaction between the two drugs.

Brd4 and CDK9 inhibitors suppress transactivation of genes induced by various inflammatory stimuli

Having established the combination of drugs to synergistically inhibit a single PRG (iNOS) by IL-1b, we next investigated whether this drug combination would also inhibit other upstream pathways leading to iNOS activation. Chondrocytes were treated with IL-1b, TNF- α , and IL-6 with IL-6 receptor (Fig1C). As expected, IL-1 β , TNF- α , or IL-6 with IL-6 receptor (~447-, 127-, and 69-fold, respectively) treatments markedly induced iNOS mRNA expression, the maximum induction from each stimulus was arbitrarily set to 100%. In cells treated with each of the three inflammatory stimuli, co-treatment with high dose of JQ1 (1000nM) or Flavopiridol (250nM) suppressed iNOS induction, as did the combined drug treatment at lower doses (Fig1C), consistent with the synergy observed above.

We next tested the effects of Cdk9 and Brd4 inhibition on the induction of additional inflammatory genes (COX2) and catabolic genes (MMP1, 3, 9, 13, and ADAMTS4) under identical experimental conditions. Similar results and synergy between the two drugs were observed (Supp. Fig1A–C). In contrast, the two drugs did not affect the expression of anabolic genes Aggrecan and Collagen IIa, which were not induced by the three inflammatory stimuli (Supp Fig1A–C). Taken together, these data suggest that multiple upstream signaling pathways and different inflammatory response genes are affected by the synergistic interactions between JQ1 and Flavopiridol, whereas non-induced genes (Agcn, ColII) are not affected.

Brd4 and CDK9 inhibitors cooperatively suppress transactivation of a broad array of inflammatory genes

To expand these observations across the entire transcriptome, we next investigated the effects of the two inhibitors by comprehensive transcriptome analysis. The gene expression profiles from human chondrocytes (n=2 donors) treated with IL-1b, in the presence or absence of single or combined inhibitors were determined. A total of 873 genes were induced >1.5-fold within 5 hours of IL-1b treatment alone. The relative expression of these IL-1b-induced genes is shown as a heat map (Fig1D). Treatment with a high dose of JQ1 or Flavopiridol alone suppressed the induction of many IL-1b-induced genes (Fig1D, lanes 2&3). However, JQ1 and Flavopiridol each preferentially suppressed a sub-set of these 873 genes, whereas the combined drug-treatment suppressed the induction of most of the 873 genes (Fig1D, lane 4). In the combined drug treatment, the gene expression profile was restored to levels closest to untreated control as determined by unsupervised hierarchical clustering analysis. These results indicate that there is a synergistic effect between JQ1 and Flavopiridol to suppress the induction of a broad spectrum of genes during the inflammatory response elicited by IL-1b.

Brd4 and CDK9 inhibitors protect cartilage explants from catabolic degradation.

PRGs regulated by CDK9 include many matrix-degrading enzymes that contribute to the pathogenesis of arthritis[21, 22]. Thus, we next tested whether Brd4 and CDK9 inhibitors

could reduce the catabolic destruction of cartilage explants treated with IL-1b. The amount of glycosaminoglycan (GAG) released into the culture media was determined as an indicator of cartilage degradation. GAG release was significantly higher in samples treated with IL-1b, whereas this increase in GAG release was not observed in samples treated with IL-1b plus JQ1 and/or Flavopiridol (Fig2B). The combined drug dose (JQ250/Flavo60) represents a four-fold reduction of each drug, and is consistent with a synergistic interaction between JQ1 and Flavopiridol. These results demonstrate that the drugs can protect cartilage tissue from degradation induced by inflammatory conditions.

Combined JQ1/Flavopiridol treatment suppresses pro-inflammatory cytokines mRNA expression after knee injury *in-vivo*

We showed that JQ1 and Flavopiridol act in synergy to prevent PRG activation in chondrocytes and cartilage explants. We next tested an *in-vivo* situation, where inflammation is induced by an ACL-rupture (ACL-R) injury in mice[27]. We examined the immediate transcription of CDK9-dependent mRNAs, as well as downstream events that contribute to osteoarthritis pathogenesis. After ACL-rupture, mice were systemically treated with either 50mg/kg JQ1 or 7.5 mg/kg Flavopiridol (high single dose groups), or with 17mg/kg JQ1 and 2.5mg/kg Flavopiridol (low combined dose group). All animals survived the injury. After the injury, we observed a rapid increase of IL-1b and IL-6 mRNAs peaking at 4 hours (Fig3A solid gray lines), followed by a decrease in both mRNAs within 24 hours after injury. The combined inhibitor significantly suppressed activation of both mRNAs at all time points (Fig3A solid black line). Single-drug treatments had intermediate effects (Fig3A dashed and dotted lines). Taken together, these data suggest that the systemic administration of both drugs reaches the target tissues and effectively suppresses injury-induced cytokine mRNA expression.

Combined inhibitor treatment suppresses synovitis after injury

Synovitis was evaluated in the joints of naive, injured, and injured animals treated with combined JQ1/Flavopiridol, three days after injury. Histological examination of the synovium revealed a marked infiltration of neutrophils and thickening of the synovial membrane cell layers in injured joints compared to naive animals (Fig3B). However, the signs of synovitis were reduced in drug-treated animals. Scoring of synovitis in all animal groups (n=6 per group) indicated a significant increase in the average score of the injured group, but the combined drug treatment reduced the score significantly (Fig3C). These results indicate that combined JQ1/Flavopiridol treatment effectively reduces synovitis in the injured knees.

Inhibitor treatments suppress MMP activity in injured knees

Since MMPs are activated under inflammatory conditions, we next investigated the activity of MMPs in the injured knees in animals treated with the two inhibitors. The MMP activity was determined using MMPsense750 probes and *in-vivo* fluorescence spectroscopy[34]. As expected, the fluorescence intensity was significantly increased 24 and 48 hours after injury, when compared to naive controls (Fig.4A). However, in all drug treatment groups, MMP activities were significantly suppressed after injury at both 24- and 48-hour time points.

These findings suggest that JQ1 and Flavopiridol, whether administered singly or together, effectively prevent activation of MMPs in the injured joints.

Inhibitor treatment reduces mechanical deterioration of articular cartilage after injury

ACL rupture injury caused a significant loss of cartilage mechanical properties at 3 and 7 days in both the medial and lateral femoral condyles as detected by AFM-nanoindentation (Fig4B). However, the loss of cartilage mechanical properties was significantly reduced in drug-treated animals at seven days after injury, in both the medial and the lateral condyles (Fig4B right panel). A similar trend was observed at the 3-day time point, although without reaching statistical significance in n=6 animals (Fig4B left panel). Given that mechanical properties represent an integrated response of cartilage composition and structure,[35] these results provide direct evidence on the effect of inhibitors in attenuating cartilage degeneration.

Inhibitor treatment reduces PTOA severity

We next examined the long-term effects of JQ1/Flavopiridol treatment on the progression of PTOA in animals. Histological analysis was performed in knees of injured animals treated with or without inhibitors at 2- and 4-weeks post-injury. Representative images of histological knee sections in each experimental group are shown (Fig5A). OARSI scoring of osteoarthritis showed that at two weeks after injury, there was a mild increase in osteoarthritis score that progressed to more severe arthritis by four weeks, with a score of 6. However, the OARSI score was significantly reduced in drug-treated animals at four weeks (score 2.8) (Fig5C), and a similar trend was observed at the 2-week timepoint although this did not reach statistical significance with n=6 animals (Fig5B). Drug treatment also significantly reduced injury-induced osteophyte area at 4-weeks (Table S2). These results indicate that combined treatment with JQ1/Flavopiridol reduces PTOA progression in our mouse model.

Discussion

Inflammation is linked to catabolic destruction of joint tissues is a significant factor in PTOA pathogenesis of. Recent advances indicate that Brd4 and CDK9 are the rate-limiting factors in activation of PRGs, a category of genes that includes the inflammatory genes.[19, 20] The CDK9 inhibitor Flavopiridol has potent anti-inflammatory effects in vitro [21, 22], and it can reduce injury-induced inflammation and the associated secondary joint damage in our ACL-rupture mouse model (unpublished data). Considering these findings, this is the first study to investigate the effects of inhibitors of Brd4 and CDK9 on suppressing inflammation and PTOA progression. We postulate that by targeting both the recruitment and the kinase activity of CDK9 with JQ1 and Flavopiridol, respectively, one could formulate a superior anti-inflammatory agent. Indeed, we observed a synergistic effect between JQ1 and Flavopiridol in suppressing inflammation-induced genes. The *in-vivo* ACL-rupture mouse model confirmed this synergistic effect. The histological OA scores highlight the alleviating effects of these drugs on joint degradation. In OA murine model, the reduction of the cartilage modulus precedes the appearance of histological OA signs.[29] AFM-nanoindentation modulus is a highly sensitive indicator of cartilage structural integrity

and early OA. The attenuated modulus reduction observed on the inhibitor-treated joints thus provides quantitative, functionally relevant evidence of the protective effects of these inhibitors on cartilage matrix degeneration.

Using a statistical design-of-experiments approach we were able to predict a synergistic effect of JQ1 and Flavopiridol on suppressing the inflammatory response. This synergy was confirmed *in-vitro* (Fig 1) and subsequently by other experiments in this study. Our data demonstrated that JQ1 and Flavopiridol, when used individually at higher doses, or in combination at lower doses, has similar effects on repressing inflammatory/catabolic mRNA expression and MMP activity in knee joints following trauma. Further research is required to reveal the precise mechanism and main gene targets that enable JQ1 and Flavopiridol to function synergistically in suppressing downstream inflammatory pathways and in reducing PTOA severity.

Both Flavopiridol and JQ1 are in clinical trials as anti-cancer drugs based on effects on cell cycle progression[36, 37]. However, both drugs have significant dose-limiting toxicities that may limit their clinical use. For example, Flavopiridol can increase rates of major tumor lysis syndrome, cytokine release syndrome, neutropenia, secretory diarrhea, and orthostatic hypotension[38]. We observed significant weight loss in mice treated with high dose Flavopiridol administered for three days, but no significant weight loss with either high dose JQ1 or the low dose combined drug treatment (Supplementary Figure 2). The reduced weight loss demonstrates the potential benefit of the combined drug treatment strategy. A limitation of the study design is that there was not an uninjured group that received drug treatment. The systemic toxicity of the combined drug treatment remains to be studied in detail, and a precise treatment window and duration need to be identified to minimize the drug exposure before clinical translation can occur.

The recent focus of post-traumatic arthritis research has been shifting toward providing a better understanding of the early events after injury. [39] The PTOA model used in this study was non-invasive mechanical rupture of the ACL. The non-invasive nature of the injury enables the study of the natural progression of the early events following injury, such as the expression levels of PRGs within the first few hours. However, ACL-rupture initiates more rapid progression to OA than other models such the destabilization of the medial meniscus (DMM).[40] Future research will determine whether the effects observed in this study apply to other OA models, and test whether this is a viable option for the human clinical context.

A further limitation of is that subchondral bone changes were not quantified. Moreover, the drug effects on pain and disability should be evaluated in future studies, as these often do not correlate with structural OA pathology.

In summary, we observed a synergistic interaction between two small-molecule inhibitors, Flavopiridol and JQ1, that act on the transcription factor CDK9 and its recruitment factor Brd4, respectively. The synergy is a promising avenue to reduce the overall effective therapeutic dose and minimize side effects. The results support that targeting mRNA transcription of primary response genes after joint injury is a viable therapeutic strategy to reduce OA severity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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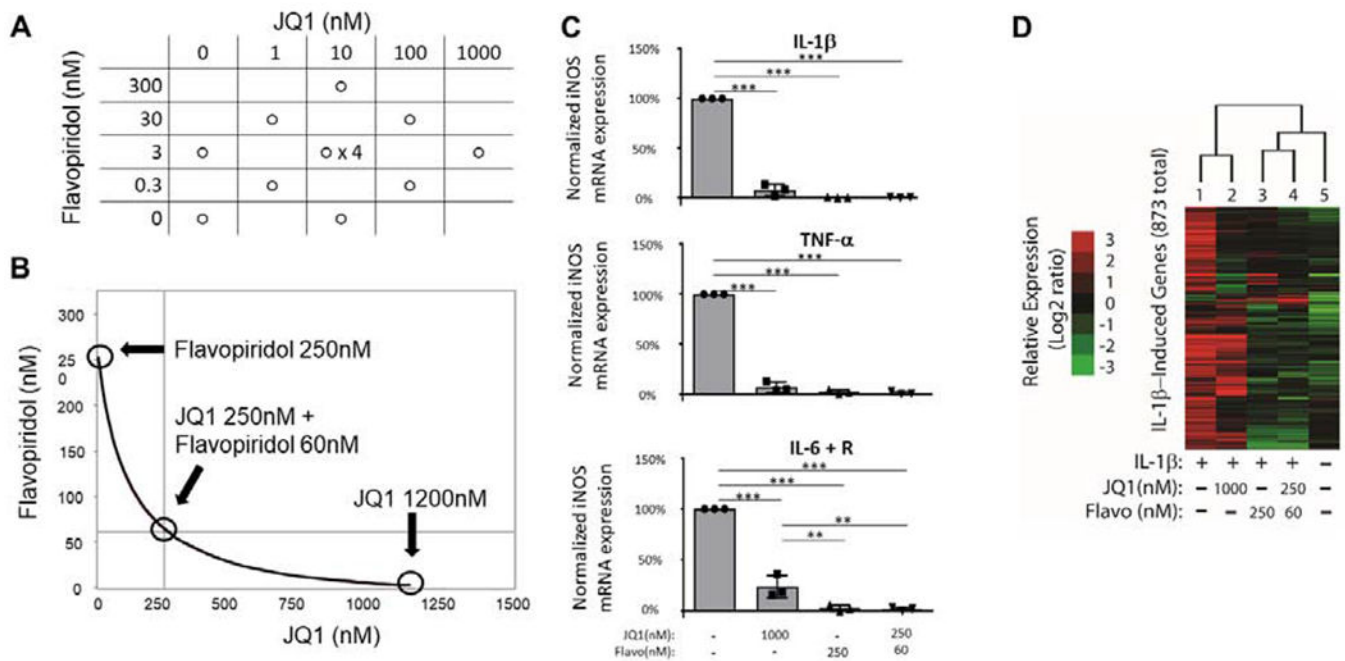


Figure 1.

A. Design-of-Experiment statistical matrix for determining optimal dosing of JQ1 and Flavopiridol. A circle represented that dosage tested in 3 separate donors (n=3) while the combination of 10nM of JQ1 and 3nM of Flavopiridol was tested 4 times in 3 separate donors (n=12).

B. Contour plot representing complete repression of IL-1 β -induced iNOS gene expression. When administered alone, 1200nM of JQ1 or 250nM of Flavopiridol, was required to achieve 100% suppression of iNOS mRNA induction. A lower combined dosage of 250nM of JQ1 and 60nM of Flavopiridol also achieved 100% iNOS suppression. The statistical model predicts 100% inhibition of the iNOS response for any combination of Flavopiridol and JQ1 that lies on the curve in Figure 1B.

C. Effects of JQ1 and Flavopiridol on iNOS mRNA expression in chondrocytes under different inflammatory stimulation. Hi J: 1200nM JQ1, Hi F: 250nM Flavopiridol, Lo Comb: 250nM JQ1 + 60nM Flavopiridol. Data were the mean \pm SD of 4 different donors, individual data points within each sample groups were represented by different symbols ***:p<0.001, **:p<0.01 for the indicated comparisons.

D. Effects of inhibitors on expression of IL-1 β -induced genes. Microarrays revealed there were 873 genes induced more than 1.5-fold by IL-1 β in human chondrocytes (n=2 donors). Relative gene expression was shown as heat map and hierarchical clustering, where green indicates lower expression and red indicates higher expression. Treatment with JQ1 and/or Flavopiridol suppressed most of the IL-1 β -induced.

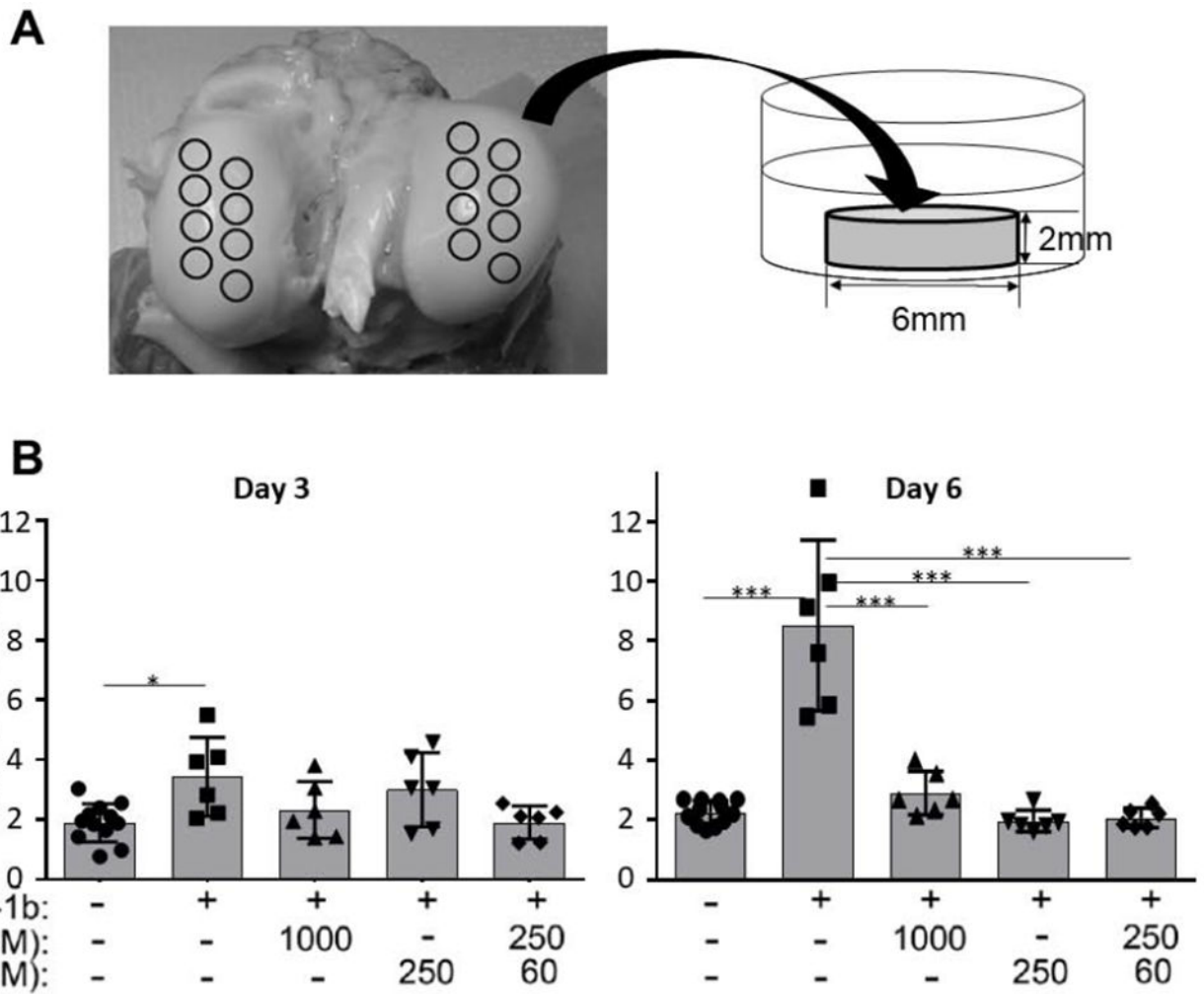


Figure 2.

A. Distal articular surface of bovine femur. Circles represented locations of harvested cartilage explants that were subsequently cultured in the media with or without IL-1b (10ng/ml).

B. GAG released into the culture media. The amounts of GAG released was determined as indicator of cartilage matrix degradation. IL-1b treatment significantly increased the GAG released, which were suppressed by JQ1 and/or Flavopiridol treatment. Values were means \pm SD (n= 6 different donors). ***:p<0.001, *:p<0.05 for the indicated comparisons.

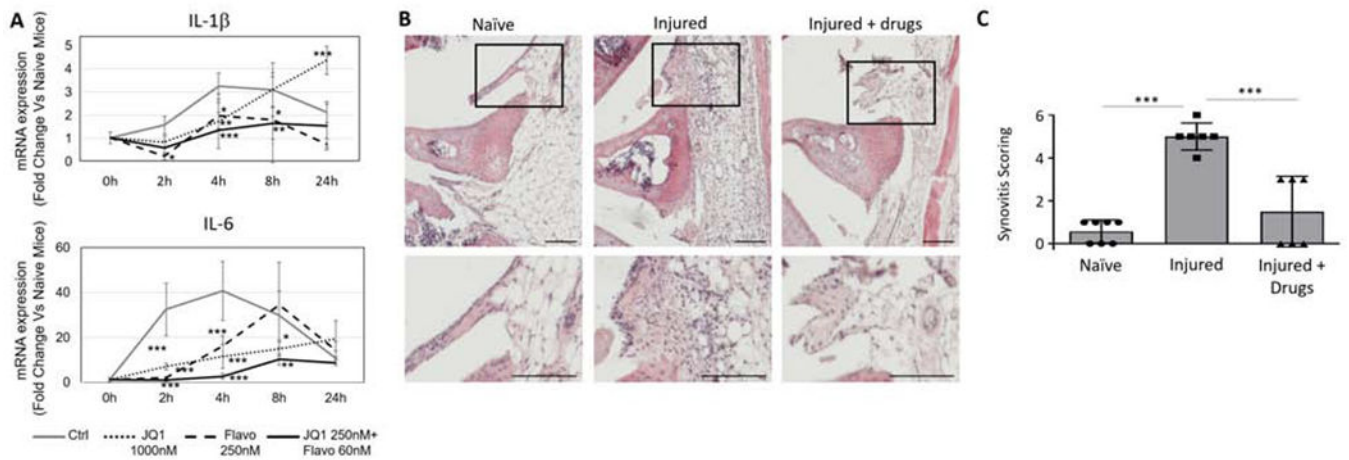


Figure 3.

A. Effects of CDK9 inhibitors on gene expression in injured knees. Mice (n=4 per group per timepoint) were subjected to ACL rupture procedure and treated with JQ1 and/or Flavopiridol. The expression of IL-1 β and IL-6 mRNAs in knee joints was determined by RT-PCR. The Y-axis represented the fold change (mean \pm SD) of mRNA expression relative to Naive controls. ***:p<0.001, **:p<0.01, *:p<0.05 against the control group (Control=gray line, JQ1=dotted black line, Flavo=dashed black line, JQ1/Flavo=solid black line).

B. Histological evaluation of synovial tissues. Representative sagittal sections of synovial tissue around meniscus of the Naive, Injured, and Injured mice treated with JQ1/Flavopiridol at 3 days post-injury. Top panels: 10X magnification. Lower panels: 40X magnification of the boxed area in each corresponding sample. Bar =50 μ m

C. The histopathological grading of synovitis. The synovitis score of the Injured group was significantly greater than that of the Naive groups, however, drug treatment significantly reduced the severity of synovitis. n = 6 per group.

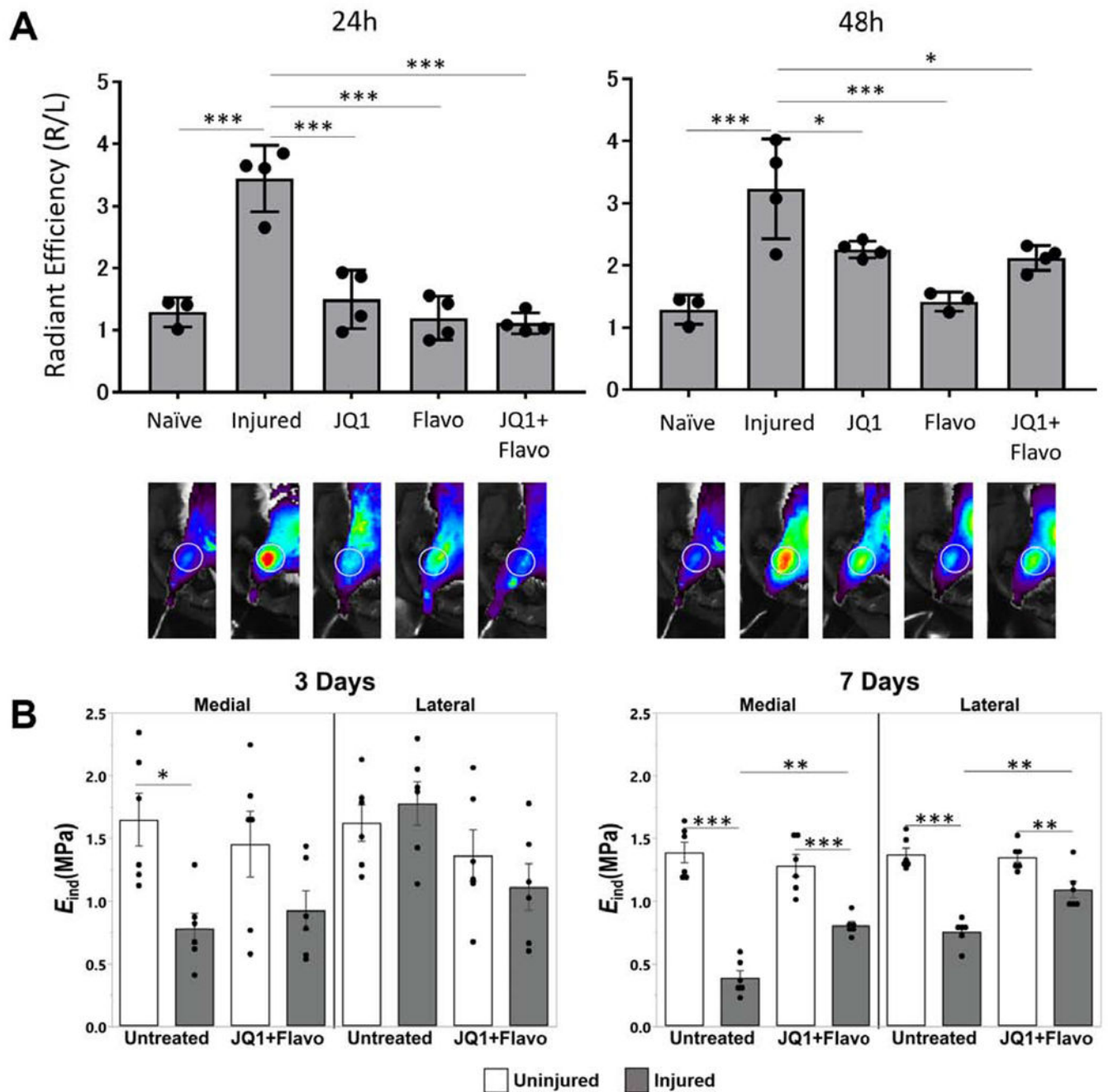


Figure 4.

A. Protease activity at injured joints treated with CDK9 inhibitors. The MMP activity in injured knee joints was determined using MMPsense 750 and in vivo fluorescence imaging. Ratio of the fluorescence intensity (radiant efficiency) of injured knee vs uninjured contralateral knee was determined for each animal ($n=4$ /group/timepoint). Values were means \pm SD. ***: $p<0.001$, *: $p<0.05$ for the indicated comparisons. Lower panels were representative images indicating the areas of interest around knee joints used for quantification of fluorescence intensity. Red color indicated an increased in fluorescence intensity.

B. Comparison of Young's modulus on the condyle surfaces between the untreated and inhibitor-treated joints at 3 and 7 days after knee injury. The left knee of each animal was used as uninjured control. Values were the mean \pm SEM of n=6 different animals per timepoint. ***:p<0.001, **: p<0.01, *:p<0.05 for the indicated comparisons via one-way ANOVA followed by Tukey-Kramer post-hoc comparison. Open bars=uninjured, gray bar=injured.

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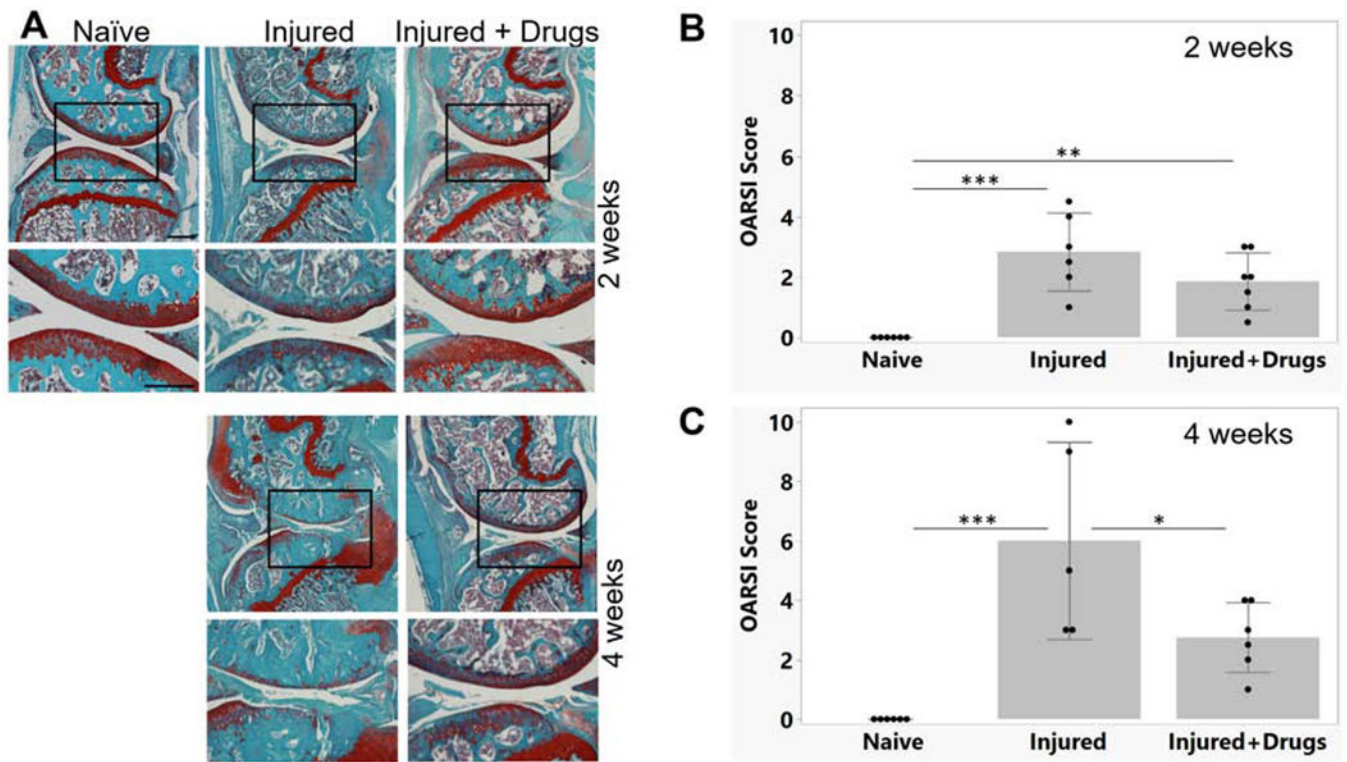


Figure 5.

Histological evidences of osteoarthritis progression.

A. Histological evaluation of OA progression in animals treated with CDK9 inhibitors. Joint integrity was evaluated by Safranin O and Fast Green staining at 2 and 4 weeks after injuries. Representative images (10X) for the Naïve, Injured, and Injured + drug treated animals were shown. The boxed area for each sample were shown below in 40X magnification.

B, C. The severity of OA was assessed by OARSI semi-quantitative scoring at 2- and 4-weeks post-injury. n=6/group, except n=5 for Injured group at 4wks, and n=7 for Injured +Drug group at 2wks..