Chondroprotective effects of Salubrinal in a mouse model of osteoarthritis

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Objectives
Salubrinal is a synthetic agent that elevates phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) and alleviates stress to the endoplasmic reticulum. Previously, we reported that in chondrocytes, Salubrinal attenuates expression and activity of matrix metalloproteinase 13 (MMP13) through downregulating nuclear factor kappa B (NFκB) signalling. We herein examine whether Salubrinal prevents the degradation of articular cartilage in a mouse model of osteoarthritis (OA).

Methods
OA was surgically induced in the left knee of female mice. Animal groups included age-matched sham control, OA placebo, and OA treated with Salubrinal or Guanabenz. Three weeks after the induction of OA, immunoblotting was performed for NFκB p65 and p-NFκB p65. At three and six weeks, the femora and tibiae were isolated and the sagittal sections were stained with Safranin O.

Results
Salubrinal suppressed the progression of OA by downregulating p-NFκB p65 and MMP13. Although Guanabenz elevates the phosphorylation level of eIF2α, it did not suppress the progression of OA.

Conclusions
Administration of Salubrinal has chondroprotective effects in arthritic joints. Salubrinal can be considered as a potential therapeutic agent for alleviating symptoms of OA.

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Strength and limitations
Strengths: This is a pre-clinical study that indicates novel treatment of patients with OA. Salubrinal is a synthetic agent that provides beneficial effects, not only by delaying the progression of OA, but also by preventing bone loss in a separate mouse model of osteoporosis.

Limitation: The observed effects on OA should be further examined using both female and male mice at different time points and dosages of Salubrinal.

Introduction
Osteoarthritis (OA) is the most common form of arthritis, resulting from multiple factors including ageing, traumatic injuries and abnormal gait biomechanics, as well as...
genetic and metabolic elements linked to obesity and inflammation.1-4 Approximately 630 million people are affected worldwide, but injured cartilage does not heal spontaneously. In many cases, cartilage defects are replaced by fibrous cartilage that exhibits significantly inferior mechanical properties.5-7 The progression of OA is represented by multiple detrimental events, starting with the death of chondrocytes, followed by the upregulation of proteolytic enzymes.8,9 Weight loss, low-impact exercise and the strengthening of muscles are generally recommended to help slow its progression. A common practice also includes administration of non-steroidal anti-inflammatory medicines, as well as injection of cortisone and hyaluronic acid.10 Surgical treatments might be an ultimate choice. However, it introduces the risk of infection and damage to surrounding structures.11,12

Pre-clinical and clinical studies have been conducted with naturally derived chemicals, synthetic agents, and biological molecules.13-17 In this study, we focused on Salubrinal (Tocris Bioscience, Bristol, United Kingdom, C21H17Cl3N4Os, 480 Da), a synthetic chemical, as a potential chondroprotective agent. Salubrinal has been shown to prevent bone loss by elevating the phosphorylation level of eukaryotic translation initiation factor 2 alpha (eIF2α).18 This elevation upregulates activating transcription factor 4 (ATF4), which is one of the key transcription factors in bone formation.19,20 Furthermore, an increase in eIF2α phosphorylation is reported to inhibit bone resorption by inactivating the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1).20,21 Because of Salubrinal’s dual beneficial role in protecting and rebuilding bone, an intriguing issue is its potential effect on cartilage. We have shown previously that Salubrinal downregulates expression and activity of matrix metalloproteinase 13 (MMP13) in C28/I2 chondrocytes.22 However, its efficacy in the treatment of OA needs to be examined.

Herein, we address a pair of questions using primary chondrocytes and a mouse model of post-traumatic OA: does Salubrinal chondroprotect the damaged articular cartilage? If so, what signalling pathway mediates its effect? Based on our previous study using C28/I2 chondrocytes, we focused on nuclear factor kappa B (NFκB) signalling and assayed MMP13 activity. We used Guanabenz (Tocris Bioscience) as a positive control as a regulator of eIF2α phosphorylation. We also evaluated the response to TNFα in primary and C28/I2 chondrocytes, and examined the efficacy of Salubrinal in a mouse model of OA using histology.23,24

Materials and Methods

Cell culture. Human primary chondrocytes (PC136121A1-C, Asterand Bioscience, Cambridge, Massachusetts) and human chondrocyte cell line, C28/I2, were cultured in DMEM/F12 and DMEM mediums, respectively, which were supplemented with 10% fetal bovine serum and antibiotics (Life Technologies, Grand Island, New York). Cells were maintained at 37°C and 5% CO2 in a humidified incubator. After ten hours of serum-free conditions, cells were incubated with 10 ng/ml TNFα (R&D Systems, Minneapolis, Minnesota) in the presence and absence of 5 μM Salubrinal or 5 μM Guanabenz (TOCRIS Bioscience, Ellisville, Missouri). One μM thapsigargin (Santa Cruz Biotechnology, Santa Cruz, California) was used as a positive control (PC) for phosphorylation of eIF2α.

A mouse model of OA and administration of Salubrinal or Guanabenz. OA was surgically induced in the left knees of 118 C57/BL6 female mice (approximately nine weeks old) by transecting the medial collateral ligament and removing the medial meniscus.23 Animal groups included age-matched sham control (CN), OA placebo, and OA treated with Salubrinal (OA + Sal). The sham surgery consisted of incision and suture. We also employed additional age-matched sham control and two OA groups treated with Guanabenz and its solvent (placebo). A total of three days after the induction of OA, Salubrinal (1.5 mg/kg) or Guanabenz (2.0 mg/kg) was administered daily into an intra-articular space, while their solvents (49.5% PEG 400 and 0.5% Tween 80 in PBS for Salubrinal; and distilled water including 5% glucose for Guanabenz) were used in the placebo groups. Mice were anaesthetised, after which injection was conducted to the intercondylar fossa from the patellar tendon in a sagittal plane to avoid potential damage to the load-bearing cartilage surface.

Western blot analysis. Human primary chondrocytes (15 minutes after addition of TNFα) and tibia cartilage (three weeks after the induction of OA) were lysed in a radio-immunoprecipitation assay buffer.22 We chose a tibia sample as they were able to be harvested with fewer potential contaminations with subchondral bone or bone marrow than femur samples. Isolated proteins were gel-fractionated and electro-transferred to Immobilon-P membranes (Millipore, Billerica, Massachusetts). The antibodies used were phosphorylated NFκB p65, NFκB p65 (Cell Signaling, Beverly, Massachusetts) and β-actin (Sigma, St Louis, Missouri). Images were taken with a luminescent image analyser (LAS-3000, Fuji Film, Tokyo, Japan), and signal intensities were quantified with Image J software (National Institute of Health, Bethesda, Maryland). Data were presented with reference to control intensities of β-actin.

Safranin O staining. Knee samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for two weeks, embedded in paraffin and sectioned at 4 μm thickness. After deparaffinisation and rehydration, slides were stained with Weigert’s iron haematoxyline solution for five minutes. They were differentiated in 1% acid–alcohol, and stained with 0.02% fast green solution. They were then rinsed in 1% acetic acid, stained in 1% Safranin O solution for 30 minutes and treated with graded ethyl alcohol and xylene.25

Histological score for symptoms of osteoarthritis. Tissue sections (approximately 50 sections per mouse) were graded...
using the scoring system described by Glasson et al. \(^{24}\)
Because of the specific procedure taken for OA induction, we focused on the histological evaluation of the medial side that exhibited more severe symptoms than the lateral side. Accordingly, the grade was assigned by two independent scorers blinded to treatment allocation (KH, AN) as follows: “0” = normal; “0.5” = loss of Safranin O without structural changes; “1” = small fibrillations without loss of cartilage; “2” = vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina; and “3” to “6” = vertical clefts/erosion to the calcified cartilage extending to < 25%, 25% to 50%, 50% to 75%, and > 75% of the articular surface, respectively.

**Determination of thickness of subchondral bone and synovial score.** To evaluate the effects of OA induction on subchondral bone, its average thickness was determined using the procedure described previously. \(^{26}\) In brief, the area of subchondral bone was measured using Image J software. The average thickness was then determined as a ratio of subchondral bone area to its weight-bearing width. We also determined synovial scores for evaluating inflammation of the synovium using the procedure described previously. \(^{27}\)

**MMP13 activity.** Proteins were isolated from primary chondrocytes or tibial cartilage samples (three weeks after the induction of OA) and lysed in a radioimmuno-precipitation assay buffer. MMP13 activity was analysed with a SensoLyte 520 MMP-13 assay kit (AnaSpec, Fremont, California) according to the manufacturer’s instruction. In brief, the lysates were incubated with APMA at 37°C for 40 minutes. The mixtures were then combined with MMP13 substrate solutions and incubated at 37°C for 30 minutes. Fluorescence intensity for MMP13 activity was determined at 485 nm (excitation) and 528 nm (emission).

**Statistical analysis.** For *in vitro* experiments, three or four independent experiments were conducted and the Student’s *t*-test was employed. For the evaluation of histological scores, thickness of subchondral bone, and synovial scores, non-parametric statistical analysis (Kruskal–Wallis test and Mann–Whitney U test) was conducted. For the interpretation of *in vivo* protein samples, one-way analysis of variance followed by Dunnett’s *post hoc* test, was conducted. Data were expressed as mean and standard deviation (SD), and statistical significance was evaluated at *p* < 0.05. The single and double asterisks indicate *p* < 0.05 and *p* < 0.01, respectively.

**Results**

**In vitro suppression of TNFα-induced NFκB phosphorylation and MMP13 activity by Salubrinal.** In response to 10 ng/ml of TNFα, primary chondrocytes (PC136121A1-C) elevated the level of phosphorylated NFκB (p-NFκB) without altering the total level of NFκB (Fig. 1a). Their incubation with TNFα also increased MMP13 activity (Fig. 1b). However, simultaneous application of 5 μM of Salubrinal with TNFα significantly reduced the level of p-NFκB as well as MMP13 activity (Figs 1a and 1b). Although both Salubrinal and Guanabenz elevate the phosphorylation level of elf2α, Guanabenz did not alter the level of p-NFκB (Fig. 1c) or MMP13 activity (Fig. 1d).

**Reduced cartilage degradation by Salubrinal into the intra-articular space.** In the mouse model of osteoarthritis, the sagittal sections stained with Safranin O indicated degradation of articular cartilage on the tibial and femoral surfaces in the knee joint (Fig. 2). The intensity of Safranin O staining decreased in the samples harvested three and six weeks after the induction of OA. However, daily administration of Salubrinal at a dose of 1.5 mg/kg from three days after the induction of OA partially restored Safranin O staining.

The histological score (0 for normal, and 6 for worst OA) revealed that Salubrinal significantly reduced OA-linked tissue degeneration (Fig. 3). In week three samples, the histological mean scores for the femoral condyle (MFC) were 0.8, SD 0.3 (control; CN), 3.8, SD 1.2 (OA placebo; OA), and 2.5, SD 0.5 (OA Salubrinal; OA + Sal) (CN vs OA, *p* < 0.01; and OA vs OA + Sal, *p* = 0.045), while the scores for the medial tibial plateau (MTP) were 0.3, SD 0.5 (CN), 2.8, SD 1.0 (OA), and 2.2, SD 0.8 (OA + Sal) (CN vs OA, *p* < 0.01; and OA vs OA + Sal, *p* = 0.530). In week six samples, the scores for MFC were 0.6, SD 0.2 (CN), 5.1, SD 0.8 (OA), and 4.0, SD 1.9 (OA + Sal) (CN vs OA, *p* < 0.01; and OA vs OA + Sal, *p* = 0.163), while the scores for MTP were 0.6, SD 0.4 (CN), 5.3, SD 0.7 (OA), and 3.7, SD 1.8 (OA + Sal) (CN vs OA, *p* < 0.01; and OA vs OA + Sal, *p* = 0.027).

Increased subchondral bone formation and marginal osteophyte development are part of the joint pathology in OA. In the mouse model of OA in this study, the thickness of subchondral bone was not altered in week three samples, but it was significantly increased in MFC and MTP in week six samples (Fig. 4a). Administration of Salubrinal did not alter the thickness of MFC and MTP at either time points. Regarding synovial inflammation, induction of OA worsened the synovial score in week three and week six samples (Fig. 4b). Salubrinal reduced the score in both samples, although statistically significant reduction was observed only in week three samples.

**In vivo suppression of OA-induced NFκB phosphorylation and MMP13 activity by Salubrinal.** Protein samples harvested from the tibial cartilage, showed upregulation of p-NFκB as well as MMP13 activity compared with the age-matched sham control (Fig. 5). Consistent with the *in vitro* result, daily administration of Salubrinal significantly reduced p-NFκB (p-NFκB/β-actin CN vs OA, *p* = 0.001; OA vs OA + Sal, *p* = 0.023; and p-NFκB/MMP13 CN vs OA, *p* = 0.285; OA vs OA + Sal, *p* = 0.042) as well as MMP13 activity (CN vs OA, *p* < 0.001; and OA vs OA + Sal, *p* < 0.001). Of note, TNFα in the *in vitro* assay did not alter the level of total NFκB, however, OA protein samples...
presented an elevated level of NFκB. We also evaluated the levels of p-eIF2α in the mouse cartilage samples as well as C28/I2 chondrocytes, in which 1 μM thapsigargin was employed as a positive control (PC) of elevation of p-eIF2α. In the cartilage samples, the significant elevation of p-eIF2α was not detected (Fig. 6a). In vitro chondrocyte samples did not also present statistically significant elevation of p-eIF2α in response to TNFα with and without 5 μM Salubrinal (Fig. 6b). Collectively, the result showed that Salubrinal-driven suppression of p-NFκB was not directly linked to the regulation of p-eIF2α.
No suppressive effects to OA progression with Guanabenz. In response to Guanabenz, the progression of OA was not detectably changed in the femur (MFC) and the tibia (MTP) both in week three and week six samples (Fig. 7). In week six samples, for example, the histological score for MFC was 0.8, SD 0.3 (CN), 5.1, SD 0.8 (OA), and 5.2, SD 0.7 (OA + Gu) (CN vs OA, p < 0.01; and OA vs OA + Gu, p = 0.772), while the score for MTP was 0.6, SD 0.4 (CN), 4.8, SD 0.7 (OA), and 4.7, SD 0.5 (OA + Gu) (CN vs OA, p < 0.01; and OA vs OA + Gu, p = 0.758). Compared with the OA control samples, administration of Guanabenz did not alter the thickness of subchondral bone of MFC and MTP at either time points (Fig. 8a). Unlike Salubrinal, which significantly reduced the synovial score in week three samples, Guanabenz did not change the synovial score both in week three and week six samples (Fig. 8b).

No significant effects on the contralateral knee. Examination of the contralateral knee, which did not receive surgery for OA induction or administration of Salubrinal or Guanabenz, revealed that no significant effects were detected on thickness of subchondral bone or histological scores.
This study demonstrates that daily administration of Salubrinal attenuates the degradation of the tibial and femoral articular cartilage and the inflammation of synovium by downregulating NFκB signalling. Salubrinal’s suppressive effects on the proteolytic activity of MMP13, as well as its elevation of the phosphorylation of NFκB p65 in the mouse model of OA, were consistent with in vitro data. The OA model in the current study induced damage in the medial collateral ligament and the medial meniscus. As post-traumatic OA may cause injuries in those regions, the results shown in this study may support the idea that Salubrinal can be applied to treat OA resulting from traumatic joint injuries.
The progression of symptoms of OA differed between the tibia and femur, and the efficacy of Salubrinal depended on time points. In response to Salubrinal, significant suppression of cartilage degradation was identified three weeks after the induction of OA in the femur, while in the tibia it was six weeks. Although the mean histological score at week three for the Salubrinal-treated OA group was similar in the femur (2.5, SD 0.5)
and the tibia (2.2, SD 0.8), the mean week three score for the OA placebo group was 3.8, SD 1.2 (femoral) and 2.8, SD 1.0 (tibial), indicating that the initial phase was more severe in the femur than in the tibia. In the week six OA placebo group, the mean score approached the worst score of six in the femur (5.1, SD 0.8) and in the tibia (5.3, SD 0.7). Significant suppression of OA symptoms was detected only in the tibia samples, although the actual mean histological score was close between the femur (4.0, SD 1.9) and the tibia (3.7, SD 1.8).

Although Salubrinal is known to reduce stress to the endoplasmic reticulum, the mechanism of Salubrinal’s action in the current study does not appear to be linked to eIF2α regulation. Guanabenz is another agent that upregulates phosphorylation of eIF2α. Unlike Salubrinal, however, Guanabenz does not alter the level of p-NFκB or reduce activity of MMP13. We have previously shown using C28/I2 chondrocytes that silencing NFκB p65 suppressed TNFα-driven upregulation of MMP13.22 In this study, in vivo and in vitro data support the notion that Salubrinal’s chondroprotection results from the down-regulation of p-NFκB (Fig. 9). We evaluated MMP13 activity, as MMP13 degrades not only collagen, but also aggrecan, and it is the most highly expressed collagenase in patients with OA.28

Regarding NFκB signalling, differential regulation of total protein levels of NFκB was observed in in vitro and in vivo experiments. Although the level of p-NFκB was elevated in both TNFα-stimulated primary chondrocytes and the OA tibial cartilage, the total protein level of NFκB was increased in the OA cartilage but not in TNFα-stimulated chondrocytes. This difference is potentially caused by the short incubation time (15 mins) with TNFα. Alternatively, in addition to TNFα, inflammatory factors such as IL1β likely contributed to the elevation of NFκB in the OA cartilage.29

Administration of Salubrinal for pre-clinical treatment of various diseases has been tested for diabetes,18 Alzheimer’s disease30 and osteoporosis.31 We, therefore, employed a mouse model of OA and showed that injection of Salubrinal into the intra-articular space delayed the progression of OA. Multiple strategies for treatment of OA have been considered, including transplantation of MSCs, antibodies directed to inflammatory cytokines, synthetic and natural chemical agents, and mechanical stimulation.25 Although Salubrinal prevents bone resorption and the stimulation of subchondral bone formation is part of the joint pathology in OA, the current mouse model of OA did not show any increase in thickness of subchondral bone in response to Salubrinal. Thus, Salubrinal is potentially able to attenuate the degradation of articular cartilage and the inflammation of synovium without causing a pathological thickening of subchondral bone.

There are several factors that might facilitate further evaluation of the efficacy of Salubrinal. First, an extended period of chondroprotection beyond three to six weeks could be evaluated by selecting proper dosages and administration frequencies. Second, daily intra-articular injection may not provide a convenient option in clinical situations. Sustained delivery of Salubrinal could be examined using implantable scaffolds or targeted nanoparticles.32 Third, with the exception of Salubrinal’s effects on chondroprotection, it is important to examine the effects on chondrogenesis including expression of matrix components such as type II collagen and aggrecan.

In conclusion, we have demonstrated that Salubrinal attenuated degradation of articular cartilage by inhibiting MMP13 activity through NFκB signalling. Further analysis regarding administration frequency and most effective dosage may warrant a future clinical trial in patients with OA.

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Supplementary material

Additional graphs showing no significant effects of osteoarthritis induction and administration of Salubrinal or Guanabenz on the contralateral knee are available with the online version of this article at www.bjj.boneandjoint.org.uk

References
