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RESEARCH

Effects of a phosphocitrate analogue on osteophyte, subchondral bone advance, and bone marrow lesions in Hartley guinea pigs

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Objectives

The objectives of this study were: 1) to examine osteophyte formation, subchondral bone advance, and bone marrow lesions (BMLs) in osteoarthritis (OA)-prone Hartley guinea pigs; and 2) to assess the disease-modifying activity of an orally administered phosphocitrate 'analogue', Carolinas Molecule-01 (CM-01).

Methods

Young Hartley guinea pigs were divided into two groups. The first group (n = 12) had drinking water and the second group (n = 9) had drinking water containing CM-01. Three guinea pigs in each group were euthanized at age six, 12, and 18 months, respectively. Three guinea pigs in the first group were euthanized aged three months as baseline control. Radiological, histological, and immunochemical examinations were performed to assess cartilage degeneration, osteophyte formation, subchondral bone advance, BMLs, and the levels of matrix metalloproteinse-13 (MMP13) protein expression in the knee joints of hind limbs.

Results

In addition to cartilage degeneration, osteophytes, subchondral bone advance, and BMLs increased with age. Subchondral bone advance was observed as early as six months, whereas BMLs and osteophytes were both observed mainly at 12 and 18 months. Fibrotic BMLs were found mostly underneath the degenerated cartilage on the medial side. In contrast, necrotic BMLs were found almost exclusively in the interspinous region. Orally administered CM-01 decreased all of these pathological changes and reduced the levels of MMP13 expression.

Conclusion

Subchondral bone may play a role in cartilage degeneration. Subchondral bone changes are early events; formation of osteophytes and BMLs are later events in the OA disease process. Carolinas Molecule-01 is a promising small molecule candidate to be tested as an oral disease-modifying drug for human OA therapy.

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Keywords: Osteoarthritis, Subchondral bone, Bone marrow lesions, MMP13, Phosphocitrate

Article focus

- The primary hypothesis of this study is that osteoarthritis development is characterized by multiple pathological bone changes in the knee joints of Hartley guinea pigs: age-dependent subchondral bone advance, osteophyte formation, and bone marrow lesions.
- The secondary hypothesis is that orally administered Carolinas Molecule-01 (CM-01) not only inhibits cartilage degeneration but also inhibits pathological bone changes.

Key messages

- Subchondral bone change is an early event in osteoarthritis development.
- Osteophytes and bone marrow lesions are later events in osteoarthritis development.
- Carolinas Molecule-01 (CM-01) is a promising small molecule candidate to be tested as an oral disease-modifying drug for osteoarthritis therapy. It exerts its disease-modifying effects by targeting pathological calcification, production of MMP13, and pathological bone changes.

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Strengths and limitations

- This is the first study to demonstrate that an orally administered phosphocitrate analogue displays a strong disease-modifying activity in the Hartley guinea pigs. The small molecule CM-01 not only inhibits cartilage degeneration but also inhibits pathological bone changes.
- We investigated the effects of Carolinas Molecule-01 (CM-01) in osteoarthritis development, but not the effects of CM-01 in reversing osteoarthritis development.
- This study was performed in guinea pigs and the results may not translate to humans. There was also no specific assessment of toxicity.

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage degeneration, synovitis, osteophyte formation, and development of bone marrow lesions (BMLs). These pathological changes are associated with severe joint pain. Osteoarthritis is one of the most prevalent causes of disability in the ageing population and has enormous economic and social consequences. However, existing non-surgical treatment options, such as non-steroidal anti-inflammatory drugs (NSAIDs), oral steroids, or steroid knee injection, only provide symptomatic relief and have no effect on the progression of cartilage degeneration, osteophyte formation, or the development of BMLs. There is a pressing need for the development of a structural OA disease-modifying drug that not only gives symptomatic relief but can also arrest the progression of OA.

The biochemical events involved in the initiation or progression of OA are poorly understood. Extracellular matrix (ECM)-degrading enzymes and inflammatory cytokines, including matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin type 1 motif 5 (ADAMTS5), interleukin-1 (IL-1), and necrosis factor alpha (TNF-α), have been implicated in OA.[1-3] Small molecules and biologics targeting MMPs, IL-1, or TNF-α have been examined for OA intervention; however, results of clinical trials with these small molecules or biologics were disappointing.[3-6] Activation and abnormal phenotypic changes of articular chondrocytes have also been implicated in OA.[7-9] Articular chondrocytes may obtain properties similar to those of terminal differentiating chondrocytes. This phenotypic change may lead to cartilage calcification, subsequent chondrocyte death, and the replacement of calcified zone cartilage by subchondral bone. However, the process of age-dependent replacement of calcified zone cartilage by subchondral bone, and its role in articular cartilage thinning or cartilage destruction, have not been examined.

Bone marrow lesions (BMLs) are characterized by decreased signal intensity on T1-weighted images and increased signal intensity on T2-weighted images. They are associated with ageing, knee injury, joint pain, and cartilage loss.[10-14] Histological examinations of knee specimens derived from OA patients indicated that BMLs seen on MRI were associated with fibrosis, necrosis, and sclerotic bone.[15,16] It has been hypothesized that fibrotic BMLs are a source of knee pain because of their association with nerve growth factor.[17] Elucidating the development of BMLs may contribute to a better understanding of their natural history and their relationship with cartilage degeneration.

Hartley guinea pigs develop OA spontaneously, and are widely used to study the pathogenesis of OA and to test disease-modifying drugs.[18-21] Although BMLs have been reported,[22-24] their types, locations, and age-dependent development have not been fully examined. Contradicting data regarding the locations and age-dependent development have been reported. Watson et al[22] reported that bone marrow cysts were observed in Hartley guinea pigs as young as two months old, and that these cysts were mainly located in the central interspinous region or the area of cruciate ligament insertions, whereas de Bri et al[23] and Tessier et al[24] reported that bone marrow cysts were observed in the medial side, and that these lesions were absent in Hartley guinea pigs younger than six months old. Therefore, further examinations are needed to determine the types, locations, and age-dependent development of BMLs in the Hartley guinea pigs. These data may advance our understanding of the OA disease process and help to develop therapeutic strategies for pain intervention, as well as preventing and treating OA.

Phosphocitrate (PC) is a powerful calcification inhibitor. It prevents soft-tissue calcification and has been reported to have no toxic side effect in rats in doses up to 150 µmol/kg/day.[25-27] We recently demonstrated that intraperitoneal injection of PC and PC analogues inhibited cartilage degeneration in Hartley guinea pig models of post-traumatic OA.[28,29] However, the prospect of PC as an oral disease-modifying drug for human OA therapy might be limited because PC contains a phosphate-oxygen atom-carbon (P-O-C) bond and five negative charges. The P-O-C bond is vulnerable to degradation by alkaline phosphatases *in vivo*, and the five negative charges make PC less permeable to intestinal membrane. In this study, we sought to examine osteophytes, subchondral bone advance, and the development of BMLs in OA-prone Hartley guinea pigs and assess the disease-modifying effects of an orally administered small molecule, Carolinas Molecule-01 (CM-01). Carolinas Molecule-01 is a small molecule that has structural similarities to PC, yet contains fewer negative charges and is resistant to alkaline phosphatases. It may be a more promising small molecule candidate than PC to be tested as an oral disease-modifying drug for human OA therapy.

Table I. Semi-quantitative histological grading scheme for subchondral bone advance

Grade	Description
0	Distance between the uppermost front of subchondral bone and tidemark is large (more than five cells away)
1	Distance between the uppermost front of subchondral bone and tidemark is moderate (four to five cells)
2	Distance between the uppermost front of subchondral bone and tidemark is small (two to three cells)
3	Distance between the uppermost front of subchondral bone and tidemark is extremely small (one cell)
4	The uppermost front of subchondral bone reaches to tidemark

Materials and Methods

Safranin-O and Fast green were obtained from Polysciences, Inc. (Warrington, Pennsylvania). An antibody specific to MMP13 was obtained from LifeSpan BioSciences, Inc. (Seattle, Washington). A secondary reagent for immunostaining (ImmPRESS reagent kit) was obtained from Vector Laboratories, Inc. (Burlingame, California). CM-01 was obtained from a commercial source and formulated in our laboratory.

Animals. Three-week-old male Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, Massachusetts) and individually housed in solid-bottom cages. Female Hartley guinea pigs have smaller body weight and develop much less severe OA than male Hartley guinea pigs with increasing age; to minimize the variation in body weight and OA severity in each age group, we chose to use only male Hartley guinea pigs. Guinea Pig Chow (No. 5025; Ralston Purina, Richmond, Indiana) and water were available *ad libitum*.

After two weeks' acclimatization, these guinea pigs were randomly divided into two groups. The first group (n = 12) had drinking water, while the second group (n = 9) had drinking water containing CM-01 (3 g/l). Three guinea pigs in each group were euthanized at the age of six, 12, and 18 months, respectively, by administration of Euthasol (Virbac Animal Health, Inc., Fort Worth, Texas). An additional three guinea pigs in the first group were euthanized aged three months and used as a baseline control. Hind limbs were collected, fixed in 10% formalin for 24 hours, and transferred to 70% ethanol until use. This study was performed according to the guidelines set out by the Institutional Animal Care and Use Committee (IACUC) of Carolinas Medical Center, which approved the animal protocol.

Radiological and histological analyses. Radiographs of the knee joints were obtained with a digital radiography system (piXarray 100; Bioptics, Inc., Tucson, Arizona). These radiographs were used to evaluate meniscal calcification and osteophytes. After dissecting the knee joints, tibial plateaus were decalcified in a formic acid/sodium citrate solution (Thermo Fisher Scientific, Fair Lawn, New Jersey) for two weeks and cut coronally in the center to produce two equal portions. Both portions were embedded in paraffin and sectioned (4 µm) with a Leica RM2025 microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Four sections from each tibial plateau were stained with Safranin-O-Fast green. These sections were graded blindly by two laboratory personnel (AS and YS) per a modified Mankin criteria.[20] Subchondral bone advance was graded per the criteria described in Table I. In total, 24 sections (12 from left knee and 12 from right knee) from each group were examined.

Thickness of articular cartilage and volume of cartilage bars or islands. The thickness of the articular cartilage was determined using ImageJ photo analysis software (version 1.8.0_45; National Institutes of Health (NIH), Bethesda, Maryland). First, the central portion of Safranin-O-Fast green-stained sections were captured with a digital camera (Sony DSC-S500; Sony Corp., Tokyo, Japan) equipped with a microscope (Nikon Optiphot-2; Nikon Corporation, Tokyo, Japan). The area of articular cartilage in the central most degenerated area was measured. Cartilage thickness was obtained by dividing the area with the length of cartilage measured. In total, 12 sections from each group were examined.

The area of articular cartilage bars or islands embedded within subchondral bone was also determined using NIH ImageJ. Briefly, cartilage bars (stained red) embedded within subchondral bone were selected using the following image thresholding and Lab colour space parameters: L, 0/255; A, 145/255; B, 0/255. After selection was completed, the area of cartilage bars (particle size setting: 0.0001 to 0.1000) in each section was automatically calculated. In total, 12 sections from each group were examined. The areas of articular cartilage bars were used as indirect indicators of the volume of cartilage bars. Normalized thicknesses of articular cartilage and normalized volume of cartilage bars were used in all analyses.

Determination of the location and numbers of bone marrow lesions. Safranin-O-Fast green-stained sections were examined to determine the types, locations, and numbers of BMLs within the epiphysis of each guinea pig. In total, 24 sections (12 from left knee and 12 from right knee) from each guinea pig group were examined by two laboratory personnel (AK and YS).

Immunostaining of MMP13. Two sections from each tibial plateau were deparaffinized with xylene and rehydrated with graded ethanol. Endogenous peroxidase activity was blocked by incubation of the sections with deionized water containing 3% H_2O_2. Non-specific binding was blocked by incubating the sections with 100 µl of 10% normal horse serum diluted in base solution (4% bovine serum albumin and 5% non-fat dry milk in phosphate-buffered saline (PBS)) for 30 minutes. These sections

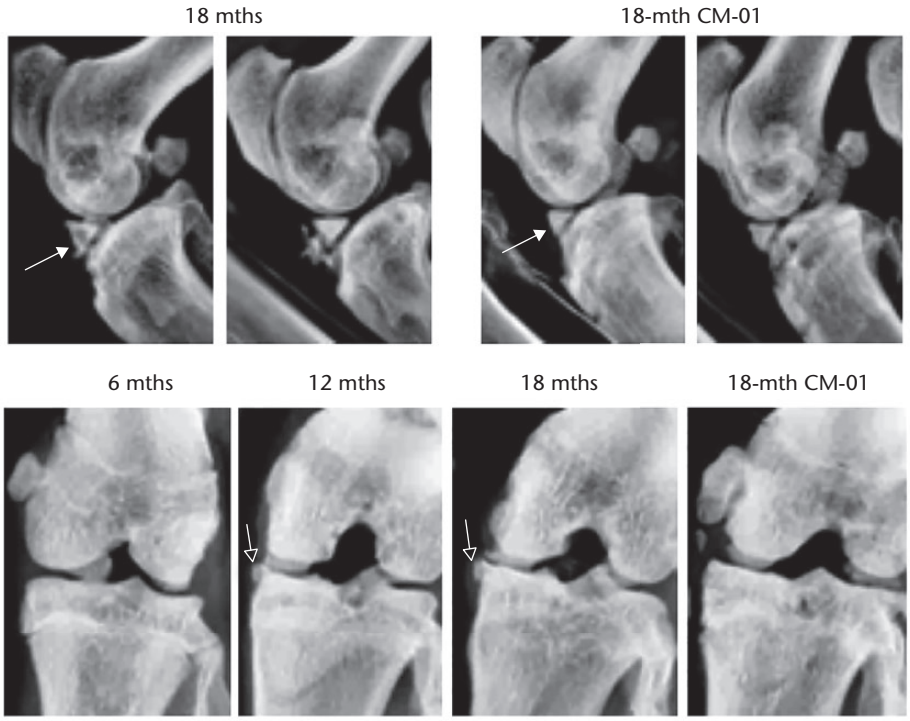

Fig. 1

Radiographs of knee joints. Carolinas Molecule-01 (CM-01) reduced the sizes of calcified anterior horn of menisci and the sizes of osteophyte. Solid arrows, calcified anterior horn of the menisci; hollow arrows, osteophytes.

were then incubated with the primary antibody (1:100 dilution) for one hour, washed three times with PBS, then followed by incubation with the secondary reagent for 30 minutes. Negative control was performed using mouse Immunoglobulin G (IgG). Slides were rinsed three times with PBS and stained with 3,3'-diaminobenzidine. Slides were counterstained with light green, dehydrated, and mounted with resinous mounting media. These slides were graded blindly by two laboratory personnel (AS and YS) on a scale of 0 to 5, as previously described.[28,30] In total, 12 sections (six from the left knee and six from the right knee) from each group were examined.

Statistical analysis. Results of cartilage thickness, area or volume of cartilage bar or islands, and the number of BMLs were presented as mean ± SD. The differences between different age groups were analyzed with one-way analysis of variance (ANOVA). The difference between untreated and CM-01-treated groups was analyzed using Student's *t*-test after passing normality and equal variance tests. Histological and immunostaining scores (variables presented as ordinal data) were also presented as the mean ± SD. The differences between different age groups were analyzed with one-way ANOVA. The difference between untreated and CM-01-treated groups was analyzed using the Mann–Whitney U test or Student's *t*-test. In all cases, $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the statistical analysis tool in SigmaPlot software, version 12 (Systat Software, Inc., San Jose, California).

Results

CM-01 inhibited meniscal calcification and osteophyte formation. Meniscal calcification, consistent with previous findings,[31,32] increased with age in the Hartley guinea pigs (data not shown). As expected, CM-01, a powerful calcification inhibitor,[29] reduced meniscal calcification (Fig. 1). The sizes of the calcified anterior horns of menisci in the CM-01-treated guinea pigs were smaller than the sizes of the calcified anterior horns of menisci in untreated control guinea pigs. Osteophyte formation also increased with age. Osteophytes were not observed in the three- and six-month-old guinea pigs but small- or moderate-sized osteophytes were observed in two 12-month-old control guinea pigs. Large-sized osteophytes were observed in all three 18-month-old control guinea pigs. In contrast, osteophytes were not observed in any of the CM-01-treated guinea pigs.

Cartilage degeneration and subchondral bone advance. Representative Safranin-O-stained sections of the medial tibial plateau are shown in Figure 2. As can be seen, minor surface irregularities and mild proteoglycan loss occurred in the superficial zone of articular cartilage in the six-month-old, compared with the three-month-old, guinea pigs. Structural lesions and proteoglycan loss became more evident and extended into the middle zone of articular cartilage in the 12-month-old guinea pigs, and these lesions further extended into the deep and calcified zones in the 18-month-old guinea pigs. Complete loss of a small or a large trunk of articular cartilage was

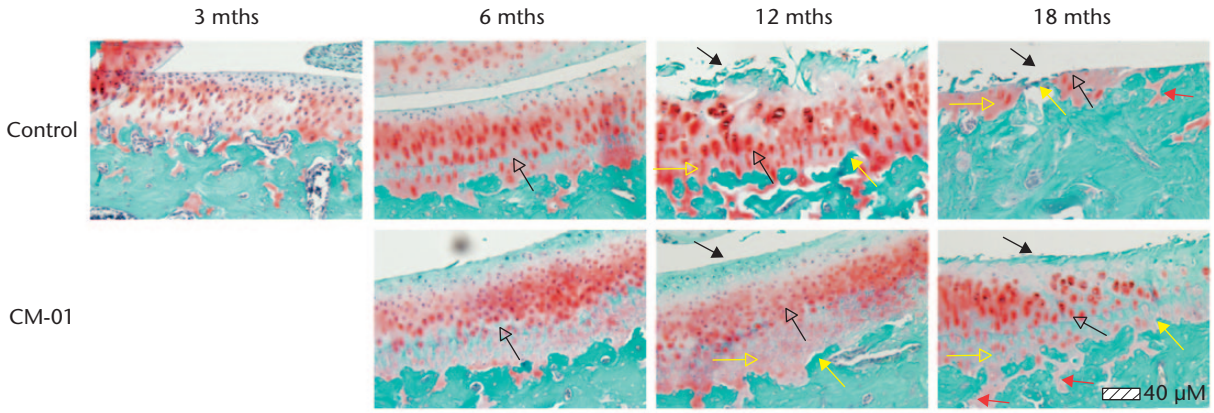

Fig. 2

Safranin-O-stained sections of medial tibial plateaus. Solid black arrows, the central most degenerated area; hollow black arrows, tidemarks; solid yellow arrows, uppermost front of subchondral bone; hollow yellow arrows, calcified zone cartilage; red arrows, articular cartilage bars or islands embedded within subchondral bone. CM-01, Carolinas Molecule-01.

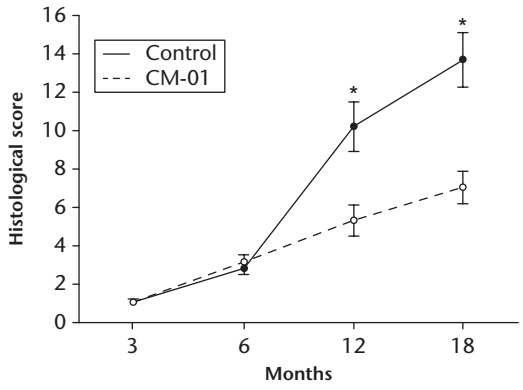

Fig. 3a

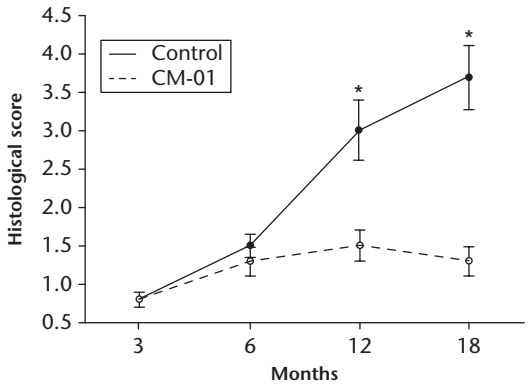

Fig. 3b

Graphs showing a) histological scores of cartilage and b) subchondral bone advance. Histological scores of articular cartilage in 18-month-old untreated guinea pigs and age-matched Carolinas Molecule-01 (CM-01)-treated guinea pigs were 13.68 (SD 1.44) and 7.02 (SD 0.04), respectively. Histological score of subchondral bone advance in 18-month-old untreated and age-matched CM-01 treated guinea pigs were 3.73 (SD 0.42) and 1.32 (SD 0.19), respectively. *$p < 0.05$, *versus* untreated control (Mann–Whitney U test).

also observed in the 18-month-old guinea pigs. The number of chondrocytes within the articular cartilage decreased with age, indicating that chondrocyte loss is associated with the progression of cartilage degeneration. In contrast, only mild surface lesions and moderate proteoglycan loss were observed in the age-matched CM-01-treated guinea pigs. The number of chondrocytes within the articular cartilage in CM-01-treated guinea pigs remained relatively unchanged with increasing age.

Several other differences were noted. The first was that the tidemarks appeared more visible in the six-month-old guinea pigs than in the three-month-old guinea pigs or the six-month-old CM-01-treated guinea pigs. Second, the uppermost front of the subchondral bone was closer to the tidemark in the untreated guinea pigs than it was in the age-matched CM-01-treated guinea pigs. Third, the calcified zone was narrower and the depth of cartilage bars or islands extending into the subchondral bone was less in the untreated guinea pigs than in the age-matched CM-01-treated guinea pigs. These differences indicated that CM-01 inhibits age-dependent cartilage calcification and the replacement of calcified zone cartilage by the subchondral bone. In other word, CM-01 inhibits the advance of subchondral bone towards the articular surface.

Histological scores of articular cartilage are provided in Figure 3. As shown, cartilage degeneration progressed slowly from three to six months, and then accelerated rapidly afterwards. Orally administered CM-01 inhibited this age-dependent cartilage degeneration significantly, resulting in a reduction of about 48% in the histological score in the 12- and 18-month-old guinea pigs ($p < 0.05$). Subchondral bone advance, different from cartilage degeneration, progressed steadily from three months to 18 months according to the histological scores. Orally administered CM-01 inhibited this advance almost completely in the 12- and 18-month-old guinea pigs ($p < 0.05$).

Articular cartilage thickness and cartilage bar volume. The thickness of the articular cartilage in the central area of

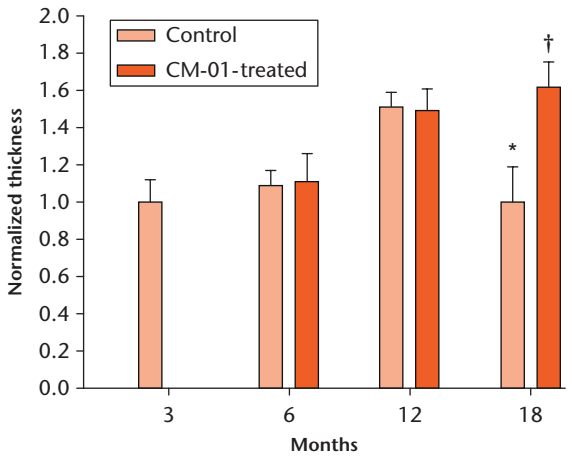

Fig. 4a

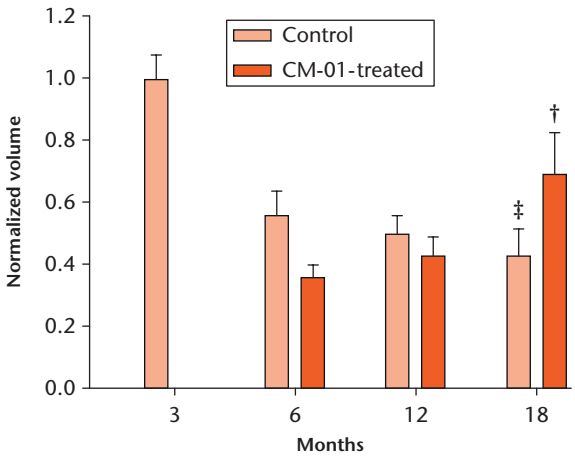

Fig. 4b

Graphs showing a) articular cartilage thickness and b) cartilage island volume in the central area of the medial tibial plateau. Light orange bars, normalized cartilage thickness and island volume in the untreated guinea pigs; dark orange bars, normalized cartilage thickness and island volume in the Carolinas Molecule-01 (CM-01)-treated guinea pigs. *$p < 0.05$, at 18 months *versus* 12 months (Student's *t*-test); †$p < 0.05$, at 18 months CM-01-treated *versus* 18 months untreated control (Student's *t*-test). ‡$p < 0.05$, at 18 months *versus* three months (Student's *t*-test).

the medial tibial plateau was thicker in the 12-month-old than in the three-month-old but it then decreased at 18 months of age (Fig. 4). The increase observed from three months to 12 months likely indicates normal age-dependent cartilage development, which was accompanied by moderate cartilage degeneration due to spontaneous OA with the increase in age. The dramatic decrease in the cartilage thickness in the 18-month-old guinea pigs compared with the 12-month-old guinea pigs was clearly caused by severe cartilage degeneration or cartilage loss due to OA progression (Fig. 2). Consistent with its OA disease-modifying activity, CM-01 inhibited articular cartilage thinning in the 18-month-old guinea pigs significantly ($p < 0.05$). The articular cartilage in the central area of the medial tibial plateau in the 18-month-old CM-01-treated guinea pigs was 62% thicker than the articular cartilage in the same area in the age-matched untreated guinea pigs.

The volumes of articular cartilage bars or islands embedded within the subchondral bone in the untreated guinea pigs decreased with age, indicating increased conversion of the articular cartilage bars into subchondral bone or increased subchondral bone advance towards the articular cartilage surface. In contrast, the volumes of articular cartilage bars in the CM-01-treated guinea pigs increased with age, especially in the 18-month-old guinea pigs. This increase was consistent with the age-dependent increase in the articular cartilage thickness in the CM-01-treated guinea pigs. In the 18-month-old CM-01-treated guinea pigs, the volume of articular cartilage bars or islands was 73% larger than the volume of articular cartilage bars or islands in the age-matched untreated guinea pigs. These findings indicate that CM-01 not only inhibits the development of articular cartilage surface lesions and damage, but also either inhibits the replacement of calcified zone articular cartilage by the subchondral bone or inhibits age-dependent subchondral bone advance towards the articular cartilage surface.

Types and locations of bone marrow lesions. Examinations of Safranin-O-Fast green-stained sections revealed the presence of three types of BMLs within the epiphysis (Fig. 5). Fibrosis (fibrotic BMLs) was characterized by fibrotic tissues embedded within the bone marrow or subchondral bone. Necrosis (necrotic BMLs) was characterized by an empty space enclosed by a thin fibrotic membrane or a fibrocartilaginous membrane. In some cases, small amounts of regenerated fibrocartilaginous tissues were present within the enclosed space. Cysts were characterized by a space filled with gel-like substances. Of the three types of BMLs, fibrosis was the most prevalent (between 70% and 75% of all BMLs) and cysts were the least prevalent (between 3% and 4% of all BMLs). More than 60% of fibrosis was found underneath the most degenerated articular cartilage on the medial side. The other 40% of fibrosis was found in the central interspinous region (area of cruciate ligament insertions). Necrosis and cysts were almost exclusively found in the central interspinous region. As shown in Figure 5, the number of BMLs increased with age. The increase was gradual between three months and 12 months, and then became rapid afterwards. Consistent with its OA disease-modifying activity, orally administered CM-01 reduced the number of BMLs significantly in the 18-month-old guinea pig compared with the age-matched untreated control ($p = 0.042$).

Levels of MMP13. Representative images of MMP13 immunostaining are provided in Figure 6. As can be seen, the levels of MMP13 protein were much higher in the 12-month-old guinea pigs than in the six-month-old

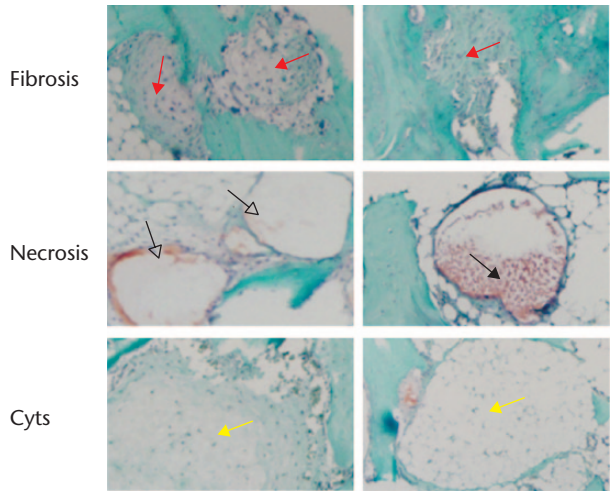

Fig. 5a

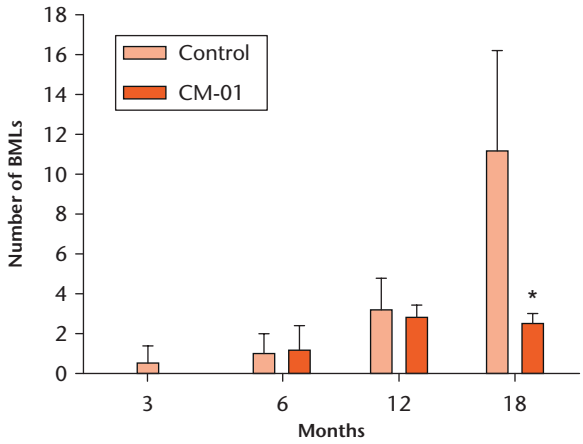

Fig. 5b

a) Three types of bone marrow lesions (BMLs) within the epiphysis: fibrosis, necrosis, and cysts. Fibrosis (fibrotic BMLs) was characterized by fibrotic tissues embedded within the bone marrow or subchondral bone (red arrows). Necrosis (necrotic BMLs) was characterized by an empty space (hollow black arrows) enclosed by a thin fibrotic membrane (stained green) or a fibrocartilaginous membrane (stained red). In some cases, small amounts of regenerated fibrocartilaginous tissues were present within the enclosed space (solid black arrow). Cysts were characterized by a space filled with gel-like substances (yellow arrows). b) Graph showing that the mean number of BMLs in the three-, six-, 12-, and 18-month-old guinea pigs were 0.5 (SD 0.87), 1 (SD 1), 3.17 (SD 1.61), and 11.17 (SD 5.01), respectively. The number of bone marrow lesions was significantly smaller in the 18-month-old Carolinas Molecule-01 (CM-01)-treated guinea pigs (2.50, SD 0.50) compared with the untreated control (11.17, SD 5.01). *$p < 0.05$, *versus* untreated control (Student's *t*-test).

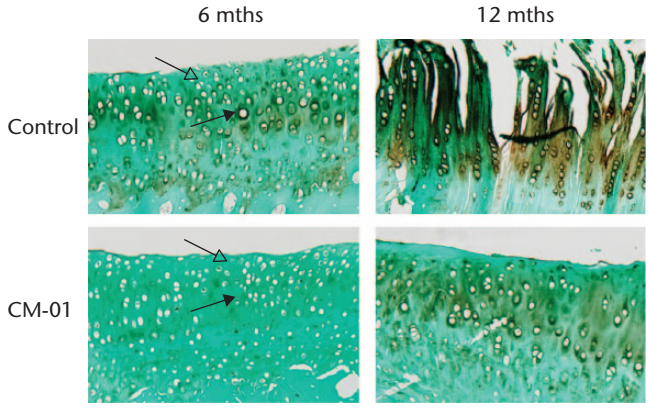

Fig. 6a

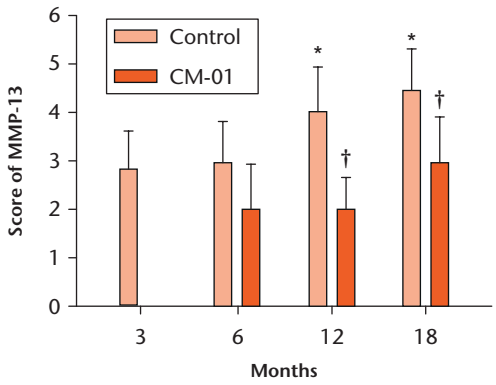

Fig. 6b

a) Immunostaining of matrix metalloproteinase-13 (MMP13) in the central area of the medial tibial plateau and immunostaining scores. Solid black arrows, middle and deep zones; hollow black arrows, superficial zone. b) Graph showing that mean immunostaining scores in the 12- and 18-month-old untreated guinea pigs, and in age-matched Carolinas Molecule-01 (CM-01)-treated guinea pigs, were 4.00 (SD 0.94), 4.45 (SD 0.86), 2.12 (SD 0.67), and 2.97 (SD 0.94), respectively. *$p < 0.05$, *versus* three-month old controls (one-way analysis of variance). †$p < 0.05$, *versus* age-matched untreated controls (Mann–Whitney U test).

guinea pigs in all three zones, indicating an age-dependent increase in the production of MMP13 by OA chondrocytes. The increase in the levels of MMP13 protein was associated with severe cartilage degeneration. Consistent with its OA disease-modifying activity, CM-01 treatment resulted in decreased levels of MMP13 protein, especially in the middle and deep zones in the six-month-old CM-01-treated guinea pigs. In the 12-month-old guinea pigs, CM-01 treatment resulted in dramatically decreased levels of MMP13 protein in all three zones (left panel). We graded these slides as described above in the Methods section. The immunostaining scores of MMP13 protein increased slightly from the age of three months to six months, and then increased significantly afterwards ($p < 0.05$) (Fig. 6). Orally administered CM-01 inhibited this age-dependent increase of MMP13 protein levels noticeably, resulting in reductions of 47% and 41% in the immunostaining score of MMP13 protein in the 12-month-old and 18-month-old guinea pigs, respectively, compared with the age-matched untreated control guinea pigs ($p < 0.05$) (Fig. 6).

Discussion

Subchondral bone consists of a subchondral bone plate, underlying trabecular bone, and bone marrow space. Although OA is characterized by progressive damage to the articular cartilage, there are significant changes in the bone of affected joints, indicating that OA is also a bone disease.[33-35] In this study, we demonstrate that, in addition to cartilage degeneration, subchondral bone

advance towards the articular cartilage was increased with age in the OA-prone Hartley guinea pigs. With the increased subchondral bone advance, the thickness of cartilage of the calcified zone and the volume of cartilage bars or islands were decreased. These findings indicate that the replacement of calcified cartilage by the subchondral bone may play a role in articular cartilage thinning or destruction. It is likely that articular chondrocytes in the calcified zone gradually recapitulate a developmental molecular programme that resembles the endochondral pathway of ossification. Carolinas Molecule-01 exerted its OA disease-modifying effects, in part, by inhibiting this molecular programme and blocking the subchondral bone advance.

Osteophytes and BMLs were also increased with age in the Hartley guinea pigs. However, unlike subchondral bone advance, which was observed in the six-month-old guinea pigs, osteophytes were not observed at the six-month stage, and were only observed in the 12- and 18-month-old guinea pigs judged by radiological examinations. These findings indicate that osteophyte formation is likely an intermediate or later event in the OA disease process, whereas subchondral bone advance is an early event. Although BMLs were present in the guinea pigs at all ages, the most dramatic increase in the number of BMLs occurred in the 18-month-old guinea pigs, indicating that a surge in the number of BMLs was a later event in the OA disease process. Our finding is consistent with the previous findings that BMLs were associated with severe cartilage loss and joint pain, both of which are characteristics of late-stage human OA.[11-14]

The most prevalent type of BML was fibrosis. The amount of fibrosis increased with age and more than 60% of fibrosis was located underneath the most degenerated articular cartilage on the medial side, indicating their association with severe cartilage degeneration. Fibrosis has been hypothesized to be a source of knee pain because of its association with severe cartilage loss.[17]. Necrosis, on the other hand, was located almost exclusively in the central interspinous region. This difference in the locations between the two types of BML suggests that the development of fibrosis is likely to be an OA-related phenomenon and the development of necrosis is likely to be an age-related phenomenon. Increased compressive stress due to severe cartilage degeneration and hypomineralization of the subchondral bone may predispose the underlying marrow or subchondral bone to fibrotic lesions, whereas accumulated tensile stress in the central interspinous region due to advanced age or overuse may predispose the underlying marrow or subchondral bone to necrotic lesions.

We have shown previously that intraperitoneal injection of PC or PC analogue inhibited cartilage degeneration in a Hartley guinea pig model of post-traumatic OA.[28,29] In this study, we show that orally administered CM-01 not only inhibited cartilage degeneration, but also inhibited meniscal calcification, subchondral bone advance, osteophyte formation, and BMLs. The reduction in articular cartilage degeneration was accompanied by a significant reduction in the level of MMP13 protein expression, a major collagen II-degrading enzyme within the articular cartilage. These findings demonstrate that CM-01 is a promising OA disease-modifying drug candidate. As osteophytes and BMLs are associated with both joint pain and severe cartilage loss, our findings indicate that CM-01 may not only delay or arrest the progression of OA but may also have a joint pain-relieving effect.

Our study has limitations. One limitation is that we only investigated the effects of CM-01 in preventing the development of OA and pathological bone changes but did not investigate the effects of CM-01 in reversing the development of OA and these pathological bone changes. Another limitation is that this study was performed in guinea pigs and the results may not translate to humans. There were also no specific assessments of toxicity. Future studies are needed to address these limitations.

In summary, our study indicates that subchondral bone may play a role in the development of cartilage degeneration. Subchondral bone advance was the earliest pathological change, cartilage structural lesions were the intermediate pathological change, while severe cartilage loss, osteophyte formation, and a dramatic surge in the number of BMLs were the later pathological changes within the knee joints in Hartley guinea pigs. Formation of fibrotic BMLs, but not necrotic BMLs, was likely an OA disease-related phenomenon. Orally administered CM-01 displays a strong inhibitory activity on all these pathological changes, and, therefore, CM-01 is a promising small molecule candidate to be tested as an oral disease-modifying drug for human OA therapy.

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Author Contributions
- Y. Sun: Designing the study, Acquiring, analyzing, and interpreting the data, Drafting and approving the manuscript.
- A. J. Kiraly: Acquiring, analyzing, and interpreting the data, Approving the manuscript.
- A. R. Sun: Acquiring, analyzing, and interpreting the data, Approving the manuscript.
- M. Cox: Acquiring the data, Approving the manuscript.
- D. R. Mauerhan: Designing the study, Drafting and approving the manuscript.
- E. N. Hanley Jr: Designing the study, Drafting and approving the manuscript.

Conflicts of Interest Statement
- None declared