Cannabinoid WIN-55,212-2 mesylate inhibits interleukin-1β induced matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression in human chondrocytes

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S U M M A R Y

Objective: Interleukin-1β (IL-1β) is involved in the up-regulation of matrix metalloproteinases (MMPs) leading to cartilage degradation. Cannabinoids are anti-inflammatory and reduce joint damage in animal models of arthritis. This study aimed to determine a mechanism whereby the synthetic cannabinoid WIN-55,212-2 mesylate (WIN-55) may inhibit cartilage degradation.

Methods: Effects of WIN-55 were studied on IL-1β stimulated production of MMP-3 and -13 and their inhibitors TIMP-1 and -2 in human chondrocytes. Chondrocytes were obtained from articular cartilage of patients undergoing total knee replacement. Chondrocytes were grown in monolayer and 3D alginate bead cultures. Real-time polymerase chain reaction (PCR) was used to determine the gene expression of MMP-3, -13, TIMP-1 and -2 and Enzyme Linked Immunosorbent Assay (ELISA) to measure the amount of MMP-3 and MMP-13 protein released into media. Immunocytochemistry was used to investigate the expression of cannabinoid receptors in chondrocyte cultures.

Results: Treatment with WIN-55 alone or in combination with IL-1β, decreased or abolished MMP-3, -13, TIMP-1 and -2 gene expression in human chondrocyte monolayer and alginate bead cultures in both a concentration and time dependent manner. WIN-55 treatment alone, and in combination with IL-1β, reduced MMP-3 and -13 protein production by chondrocytes cultured in alginate beads. Immunocytochemistry demonstrated the expression of cannabinoid receptors in chondrocyte cultures.

Conclusion: Cannabinoid WIN-55 can reduce both basal and IL-1β stimulated gene and protein expression of MMP-3 and -13. However WIN-55 also decreased basal levels of TIMP-1 and -2 mRNA. These actions of WIN-55 suggest a mechanism by which cannabinoids may act to prevent cartilage breakdown in arthritis.

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Introduction

Osteoarthritis (OA) and rheumatoid arthritis (RA) are debilitating joint diseases, and although they have a different aetiology a key pathological feature of both is the loss of articular cartilage. Cannabis-based medicine Sativex has been shown to have analgesic effects and to suppress disease activity in patients with RA.

Cannabinoids also have anti-inflammatory effects and reduce joint damage in animal models of arthritis. In vitro studies have shown that cannabinoids reduce cytokine production from RA fibroblasts and the release of matrix metalloproteinases (MMPs) from fibroblast-like synovial cells. Cannabinoids also have direct effects on cartilage extracellular matrix (ECM) breakdown; reducing interleukin 1 (IL-1) induced proteoglycan and collagen degradation in bovine cartilage. There is thus increasing evidence to suggest that cannabinoids have chondroprotective effects and may be of value in the treatment of arthritis.

During OA and RA there is a shift in the equilibrium between catabolic and anabolic activities. As a result the breakdown of collagen and proteoglycans may exceed the rate of synthesis of new matrix molecules resulting in cartilage degradation. Another contributing factor in cartilage breakdown in OA and RA is an
increase in inflammatory cytokines particularly IL-1 and tumour necrosis factor (TNF) produced by the articular chondrocytes or cells of the synovium31. This results in an increase in MMPs particularly MMP-3 and MMP-13, which are expressed in RA and OA cartilage and synovial tissue12–20, which is not accompanied by an increase in their tissue inhibitors of matrix metalloproteinases (TIMPs)21,22 resulting in a net increase in MMPs. MMP inhibition has been proposed as a possible mechanism to prevent breakdown of cartilage tissue in arthritis, provided the necessary functional specificity can be achieved23.

To investigate further the potential of cannabinoids to regulate cartilage breakdown we have studied the effects of synthetic cannabinooid WIN-55,212-2 mesylate (WIN-55) on basal and IL-1β stimulated MMP-3, -13, TIMP-1 and -2 expression in human articular chondrocytes from human OA cartilage tissue in monolayer and 3D alginate bead culture. WIN-55 is an agonist at the classical cannabinoid receptors, cannabinoid receptor 1 and 2 (CB1 and 2), but also has been shown to activate other receptors including peroxisome proliferator activated receptors alpha and gamma (PPARα and γ)24–26. Thus the expression of these receptors in cultured OA chondrocytes was determined to identify potential targets of WIN-55.

Materials and methods

Human cartilage tissue

Human chondrocytes were obtained from articular cartilage (n = 9) removed from patients with symptomatic OA at the time of total knee replacement (Supplementary Table 1). Cartilage was obtained under the National Research Ethics Service approval held by the Sheffield Musculoskeletal Biobank. All patients provided written, informed consent prior to participation. Cartilage blocks were taken from each anatomic compartment within the knee (n = 6–7) (medial and lateral tibio-femoral and patello-femoral compartments). Cartilage tissue was graded macroscopically 0–4 using the Outerbridge classification27. Chondrocytes were isolated from grade 0 (n = 3), grade 2 (n = 6) and grade 3 (n = 5) cartilage tissue as representative of non-degenerate, low degenerate and intermediate degenerate cartilage tissue. Cartilage from grade 4 severe degenerate tissue was not used in the study as the cell yield obtained was not sufficient.

Isolation of human chondrocytes

Cartilage tissue was digested in 0.25% trypsin (Sigma—Aldrich, Poole, UK) at 37°C for 30 min followed by digestion in 3 mg/ml collagenase type I (Sigma—Aldrich, Poole UK) in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1:1) (Gibco, Paisley, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Paisley, UK), 2 mM glutamine (Gibco, Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Paisley, UK), 2.5 µg/ml amphotericin B (Sigma—Aldrich, Poole, UK), and 50 µg/ml ascorbic acid (Sigma—Aldrich, Poole, UK) (complete media), at 37°C for 16 h. Cells were passed through a 70 µm cell strainer and washed twice in DMEM/P12 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 50 µg/ml ascorbic acid (serum free media). Cells were counted and the viability checked using trypan blue staining. Chondrocytes were cultured in monolayer in complete media in a humidified atmosphere of 5% CO₂ at 37°C and harvested at passage 2.

Monolayer culture

Chondrocytes harvested at passage 2 were seeded at a cell density of 1 × 10⁶ cells/well in a six well plate and maintained in complete media in a humidified atmosphere of 5% CO₂ at 37°C for 24 h prior to treatment to allow cells to adhere to the cell culture plate.

Alginate bead culture

When cultured in monolayer, chondrocytes dedifferentiate into fibroblast like cells28–30, therefore alginate beads were used to redifferentiate the chondrocytes to their native phenotype. Chondrocytes harvested at passage 2 were encapsulated in alginate beads at a cell density of 2 × 10⁶ cells/ml, as previously described31. Alginate beads were cultured in complete media in a humidified atmosphere of 5% CO₂ at 37°C and the media changed every other day. Chondrocytes were redifferentiated in alginate beads for 4 weeks prior to treatment.

Cytotoxicity studies

The CellTiter 96™ AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Southampton, UK) was used to determine the effects of 10 µM WIN-55 on the cell viability of human chondrocytes according to the manufacturer’s instructions.

Cannabinoid WIN-55 and IL-1β treatments

Prior to stimulation complete media was removed, cells washed with serum free media and serum free media containing 500 µg/ml bovine serum albumin (BSA) (Sigma—Aldrich, Poole, UK) added. Chondrocytes cultured in monolayer were unstimulated or stimulated with 10 ng/ml IL-1β (Peprotech, London, UK) with and without 1 µM, 2.5 µM, 5 µM, 7.5 µM and 10 µM WIN-55 (Sigma—Aldrich, Poole, UK) for 48 h at 37°C. Chondrocytes in monolayer were treated with 10 µM WIN-55 for 3, 6, 24 and 48 h at 37°C. Chondrocytes cultured in alginate beads were unstimulated or stimulated with 10 ng/ml IL-1β with and without 10 µM WIN-55. Dimethyl sulfoxide (DMSO) (Sigma—Aldrich, Poole, UK) was used as a vehicle control at 0.1% (equivalent to that present in 10 µM WIN-55).

MMP-3 and -13 Enzyme Linked Immunosorbent Assays (ELISAs)

ELISAs (R&D Systems, Abingdon, UK) were used according to the manufacturer’s instructions to measure pro and active MMP-3 protein expression (ng/ml) and pro MMP-13 protein expression (pg/ml) in alginate bead conditioned culture media following treatment with 10 µM WIN-55 with and without 10 ng/ml IL-1β stimulation for 48 h.

RNA extraction from monolayers

For each of the three patient samples obtained from Outerbridge grade 0, 2 and 3 (n = 9), patient samples were tested in triplicate. Following treatments, media was removed, cells washed in 1xPBS and RNA extracted in 1 ml of TRIzol reagent (Invitrogen, Paisley, UK) following the manufacturer’s instructions. RNA was resuspended in 14 µl sterile deionised water.

RNA extraction from alginate beads

For each of the three patient samples obtained from Outerbridge grade 0, 2 and 3 (n = 9), patient samples were tested in triplicate. RNA was extracted from alginate beads using two beads per extraction as described previously31. The resulting RNA was then resuspended in 100 µl of sterile deionised water and the RNA was purified using RNeasy easy clean up columns (Qiagen, Crawley, UK) according to the manufacturer’s instructions and RNA eluted in 14 µl of sterile deionised water.
Reverse transcription–real-time polymerase chain reaction (qRT-PCR)

cDNA was formed as described previously\(^{21}\). Real-time PCR was used to investigate MMP-3, -13, TIMP-1, and -2 gene expression using Applied Biosystems Taqman Gene Expression Assays (Table I). The reactions were performed for 40 cycles using Taqman Fast Universal PCR Master Mix (Applied Biosystems, Paisley, UK) on an ABI StepOnePlus real-time PCR machine (Applied Biosystems, Paisley, UK). The data were collected and the fold changes in gene expression analysed using the 2\(^{-\Delta\Delta Ct}\) method\(^{21}\). Gene expression was normalised to housekeeping genes GAPDH and 18S together with untreated controls.

Cannabinoid receptor expression in OA chondrocytes

Chondrocytes isolated from four patient samples of grade 2–3 cartilage at passage 2 were plated at a density of 1 \(\times\) 10\(^5\) cells in eight well chamber slides and allowed to adhere overnight. Chondrocytes were fixed in 4% formalin and endogenous peroxidases were quenched. Following 0.01% chymotrypsin antigen retrieval at 37°C for 20 min cells were blocked in 25% v/v goat serum/BSA. Cells were incubated with rabbit polyclonal antibodies (Abcam) overnight at 4°C against CB1 (1/100), CB2 (1/50), PPAR\(\gamma\) (1/150) and PPAR\(\gamma\) (1/50). A rabbit isotype IgG antibody (Abcam) was used as negative control. Following washing cells were incubated with biotinylated goat anti-rabbit secondary antibody (1/300; Abcam) and binding detected using streptavidin–biotinylated horseradish peroxide complex (Vector Laboratories, Peterborough, UK) with 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma–Aldrich). Cells were counterstained using Mayer’s Haematoxylin (Leica Microsystems, Milton Keynes, UK) dehydrated, cleared and mounted in Pertex (Leica Microsystems). Immunopositivity was visualised and images captured using an Olympus BX60 microscope and QCapture Pro v8.0 software (Media Cybernetics, Marlow, UK).

Statistical analysis

Data was shown to be non-parametric via a Shapiro–Wilks test hence statistical significance between DMSO vehicle control and treatment groups was determined using the non-parametric Kruskall–Wallis multiple comparisons test and Conover-Inman post hoc test; \(P < 0.05\) was considered statistically significant for real-time PCR and ELISA analysis. All statistical analysis was performed using StatsDirect. All data analysis was performed using individual replicates for each treatment group.

**Results**

**Cytotoxicity studies**

Cytotoxicity studies showed that WIN-55 was not toxic to chondrocytes at the concentrations used (Data not shown).

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<thead>
<tr>
<th>Taqman gene expression assay</th>
<th>Assay ID</th>
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<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
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</tr>
<tr>
<td>18S</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Hs00968305_m1</td>
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<tr>
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</tr>
<tr>
<td>TIMP-1</td>
<td>Hs00171558_m1</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Hs00234278_m1</td>
</tr>
</tbody>
</table>

**Time-course and concentration dependent effects of WIN-55 on MMP-3, MMP-13, TIMP-1 and TIMP-2 gene expression in monolayer**

Chondrocytes were treated with WIN-55 (10 \(\mu\)M) for 3, 6, 24 and 48 h to determine the time point at which a maximum decrease in gene expression was observed. MMP-3, -13, TIMP-1 and -2 gene expression was decreased in a time-dependent manner, the largest decrease was observed at 48 h [Supplementary Fig. 1(A–D)]; therefore this incubation time was used for all subsequent treatments. Gene expression of MMP-3, -13, TIMP-1 and -2 was decreased in a WIN-55 concentration dependent manner both with and without IL-1\(\beta\) (10 ng/ml) stimulation [Supplementary Fig. 2(A–D)]. A significant decrease in gene expression was observed at concentrations \(\geq 2.5 \mu\)M WIN-55 compared to DMSO (0.1%) vehicle controls [Supplementary Fig. 2(A–D)]. WIN-55 was used at 10 \(\mu\)M in all following treatments, as this was the maximal inhibitory non-toxic concentration.

**Effects of DMSO in monolayer and alginate bead culture**

No differences in gene or protein expression were observed between untreated cells compared to cells treated with 0.1% DMSO and 10 ng/ml IL-1\(\beta\) treatment in combination with 0.1% DMSO compared to IL-1\(\beta\) treatment alone (Data not shown). Therefore 10 ng/ml IL-1\(\beta\) in combination with 0.1% DMSO and 0.1% DMSO controls were used for all real-time PCR and ELISA analysis.

**Effect of WIN-55 on IL-1\(\beta\) induced MMP-3 and MMP-13 gene expression in monolayer**

In order to determine the effects of WIN-55 on MMPs in the presence of the inflammatory cytokine IL-1\(\beta\), real-time PCR was used to measure the gene expression of MMP-3 and -13 in chondrocytes obtained from macroscopically graded OA cartilage. For this purpose chondrocytes were stimulated with IL-1\(\beta\) to induce MMP expression and co-treated with WIN-55. The effects of WIN-55 alone were also investigated. IL-1\(\beta\) stimulation significantly induced MMP-3 gene expression in chondrocytes isolated from grade 0 \((P < 0.0001)\), 2 \((P < 0.0001)\) and 3 \((P < 0.0001)\) cartilage and MMP-13 in chondrocytes isolated from grade 0 \((P < 0.0001)\), grade 2 \((P = 0.02)\) and grade 3 cartilage \((P < 0.0001)\) compared to DMSO vehicle control [Fig. 1(A–F)]. Treatment with WIN-55 in combination with IL-1\(\beta\) significantly reduced MMP-3 and -13 gene expression in chondrocytes derived from grade 0 \((P < 0.0001)\), 2 \((P < 0.0001)\) and 3 \((P < 0.0001)\) cartilage compared to IL-1\(\beta\) stimulation alone [Fig. 1(A–F)]. WIN-55 treatment in combination with IL-1\(\beta\) also significantly reduced MMP-3 gene expression in chondrocytes isolated from grade 0 \((P < 0.0001)\), 2 \((P < 0.0001)\) and 3 \((P < 0.0001)\) cartilage and MMP-13 gene expression in cartilage from grade 0 \((P < 0.0001)\), grade 2 \((P = 0.01)\) and grade 3 \((P < 0.0001)\) compared to DMSO vehicle control [Fig. 1(A–F)]. WIN-55 treatment alone significantly reduced MMP-3 gene expression in grade 0 \((P < 0.0001)\), 2 \((P < 0.0001)\) and 3 \((P < 0.0001)\) cartilage derived chondrocytes below basal levels compared to DMSO vehicle control [Fig. 1(A–C)]. WIN-55 treatment alone also significantly decreased MMP-13 gene expression in grade 0 \((P = 0.002)\) and 3 \((P < 0.0001)\) cartilage derived chondrocytes compared to DMSO control [Figs. 1(D) and 1(F)]. MMP-13 was only expressed in two samples in chondrocytes isolated from grade 2 cartilage following WIN-55 treatment therefore statistical analysis could not be performed [Fig. 1(E)].
Fig. 1. A–C. The effect of WIN-55 on IL-1β induced MMP-3 gene expression in human OA chondrocytes isolated from grade 0, 2 and 3 cartilage and cultured in monolayer. D–F The effect of WIN-55 on IL-1β induced MMP-13 gene expression in human OA chondrocytes isolated from grade 0, 2 and 3 cartilage and cultured in monolayer. Each point represents the average of the repeats per patient. IL-1β (10 ng/ml) induced the gene expression of MMP-3 and -13. Following WIN-55 (10 μM) treatment in combination with IL-1β (10 ng/ml) MMP-3 and -13 gene expression was decreased. WIN-55 (10 μM) alone decreased MMP-3 and -13 gene expression. ***P < 0.001, **P < 0.01 compared to DMSO (0.1 %) vehicle control and +++P < 0.001 compared to IL-1β (10 ng/ml) treatment. N = 9 samples for each treatment group, obtained from three separate patient samples for each grade of tissue (0, 2 and 3) tested in triplicate.
Fig. 2. A–C. The effect of WIN-55 on IL-1β induced MMP-3 gene expression in human chondrocytes isolated from grade 0, 2 and 3 cartilage and cultured in alginate beads. D–F. The effect of WIN-55 on IL-1β induced MMP-13 gene expression in human OA chondrocytes isolated from grade 0, 2 and 3 cartilage and cultured in alginate beads. Each point represents the average of the repeats per patient. IL-1β (10 ng/ml) induced the gene expression of MMP-3 and -13, following WIN-55 (10 μM) treatment in combination with IL-1β (10 ng/ml). MMP-3 and -13 gene expression was decreased or abolished. WIN-55 (10 μM) alone decreased or abolished MMP-3 and -13 gene expression. *P < 0.05, **P < 0.01, ***P < 0.001 compared to DMSO vehicle control and +++P < 0.001 compared to IL-1β treatment. N = 9 samples for each treatment group, obtained from three separate patient samples for each grade of tissue (0, 2 and 3) tested in triplicate.
The effects of WIN-55 on IL-1β induced MMP-3 and MMP-13 gene expression were determined in chondrocytes cultured in alginate beads in order to determine gene expression in chondrocytes following redifferentiation back to their native phenotype. IL-1β stimulation significantly induced MMP-3 gene expression in chondrocytes from grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage and MMP-13 gene expression in grade
0 ($P = 0.018$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage derived chondrocytes compared to DMSO vehicle control [Fig. 2(A–F)]. Similarly WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 gene expression in grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage derived chondrocytes and MMP-13 gene expression in grade 0 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage derived chondrocytes compared to IL-1β stimulation alone [Fig. 2(A–D) and (F)]. MMP-13 was not expressed in chondrocytes from grade 2 cartilage treated with IL-1β in combination with WIN-55 [Fig. 2(E)]. WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 ($P = 0.03$) and -13 ($P = 0.003$) gene expression in chondrocytes from grade 0 cartilage compared to DMSO vehicle control [Fig. 2(A) and (D)]. WIN-55 treatment in combination with IL-1β reduced MMP-3 gene expression in

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**Fig. 4.** A–C. The effect of WIN-55 on TIMP-1 gene expression in human OA chondrocytes isolated from grade 0, 2 and 3 cartilages and cultured in alginate beads. D–F. The effect of WIN-55 on TIMP-2 gene expression in OA human chondrocytes isolated from grade 0, 2 and 3 cartilages and cultured in alginate beads. Each point represents the average of the repeats per patient. IL-1β (10 ng/ml) stimulation increased TIMP-1 gene expression and decreased TIMP-2 gene expression below basal levels. WIN-55 treatment (10 μM) both alone and in combination with IL-1β (10 ng/ml) reduced TIMP-1 and -2 gene expression. *P < 0.05, **P < 0.01, ***P < 0.001 compared to DMSO vehicle control and ****P < 0.001 compared to IL-1β treatment. N = 9 samples for each treatment group, obtained from 3 separate patient samples for each grade of tissue (0, 2 and 3) tested in triplicate.
chondrocytes from grade 2 ($P = 0.11$) cartilage and both MMP-3 ($P = 0.58$) and -13 ($P = 0.11$) gene expression in chondrocytes from grade 3 cartilage compared to DMSO vehicle control; however this was not significant [Fig. 2(B), (C) and (F)]. There was no significant difference from basal levels of MMP-3 gene expression in grade 0 ($P = 0.052$) and 3 ($P = 0.16$) cartilage derived chondrocytes when treated with WIN-55 alone [Fig. 2(A and C)]. MMP-3 was not expressed in grade 2 cartilage chondrocytes treated with WIN-55 alone [Fig. 2(B)]. MMP-13 was abolished in chondrocytes from grade 2 and 3 cartilage treated with WIN-55 alone [Fig. 2(D–F)].

Effects of WIN-55 on IL-1β induced TIMP-1 and TIMP-2 gene expression in monolayer

In order to determine the effects of WIN-55 on the inhibitors of MMP-3 and -13 the gene expression of TIMP-1 and -2 was investigated following WIN-55 treatment both alone and in combination with IL-1β. IL-1β stimulation had no significant effect on TIMP-1 gene expression in chondrocytes derived from grade 0 ($P = 0.75$), 2 ($P = 0.60$) and 3 ($P = 0.12$) cartilage or TIMP-2 gene expression in chondrocytes isolated from grades 0 ($P = 0.77$), 2 ($P = 0.49$) and 3 ($P = 0.98$) cartilage [Fig. 3(A–F)]. However, WIN-55 treatment in combination with IL-1β resulted in a significant decrease in TIMP-1 gene expression compared to DMSO vehicle control and IL-1β stimulated chondrocytes derived from grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P = 0.0001$) cartilage [Fig. 3(A–C)]. WIN-55 alone also significantly reduced TIMP-1 gene expression in chondrocytes derived from grade 0 ($P < 0.0001$) and 2 ($P < 0.0001$) cartilage below basal levels [Fig. 3(A) and (B)]. TIMP-1 gene expression was decreased in chondrocytes derived from grade 3 cartilage following WIN-55 treatment however this was not significant ($P = 0.11$) [Fig. 3(C)]. TIMP-2 gene expression was significantly reduced following WIN-55 treatment in combination with IL-1β compared to DMSO vehicle control and IL-1β stimulation in chondrocytes derived from grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage [Fig. 3(D–F)]; WIN-55 alone significantly reduced the gene expression of TIMP-2 below basal levels in chondrocytes isolated from grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage [Fig. 3(D–F)].

Effects of WIN-55 on IL-1β induced TIMP-1 and TIMP-2 gene expression on chondrocytes cultured in alginate beads

TIMP-1 gene expression was significantly increased in grade 2 ($P < 0.0009$) and 3 cartilage derived chondrocytes ($P = 0.02$) following IL-1β stimulation [Fig. 4(A)] but not in grade 0 ($P = 0.24$) cartilage chondrocytes compared to DMSO vehicle control [Fig. 4(A)]. In contrast TIMP-2 gene expression was significantly decreased following IL-1β treatment in chondrocytes extracted from grade 0 ($P = 0.009$), 2 ($P = 0.001$) and 3 ($P < 0.0001$) cartilage compared to DMSO vehicle control [Fig. 4(D–F)]. WIN-55 treatment in combination with IL-1β resulted in a significant decrease in TIMP-1 gene expression in chondrocytes derived from grades 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$), cartilage and TIMP-2 gene expression in chondrocytes isolated from grades 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0007$), cartilage compared to IL-1β stimulation [Fig. 4(A–F)]. WIN-55 treatment in combination with IL-1β also resulted in a significant decrease in TIMP-1 gene expression in chondrocytes derived from grades 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$), cartilage and TIMP-2 gene expression in chondrocytes isolated from grades 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage compared to DMSO control [Fig. 4(A–F)]. WIN-55 treatment alone significantly reduced both TIMP-1 gene expression in chondrocytes derived from grade 0 ($P = 0.0003$), 2 ($P < 0.0001$) and 3 ($P = 0.0003$) cartilage below basal levels [Fig. 4(A–C)]. WIN-55 treatment alone significantly reduced both TIMP-2 gene expression in chondrocytes derived from grade 0 ($P < 0.0001$), 2 ($P < 0.0001$) and 3 ($P < 0.0001$) cartilage below basal levels [Fig. 4(D–F)].

The effects of WIN-55 on MMP-3 and -13 protein expression

In order to determine whether WIN-55 also inhibited MMP-3 and -13 expression at the protein level, pro and active MMP-3 and pro MMP-13 were measured in culture media using ELISA. Culture media obtained from chondrocytes isolated from grade 3 cartilage which had been cultured in alginate beads were used, as these demonstrated clear gene expression effects. Following stimulation of chondrocytes with IL-1β there was a significant increase in MMP-3 ($P < 0.0001$) and -13 ($P = 0.046$) protein release into the media (Table II). Treatment of chondrocytes with WIN-55 in combination with IL-1β significantly reduced both MMP-3 ($P = 0.0007$) and -13 ($P = 0.0005$) protein compared to IL-1β treatment alone (Table II). WIN-55 treatment alone significantly reduced MMP-3 protein release to below basal levels ($P = 0.04$) and MMP-13 ($P = 0.38$) protein levels remained at basal level (Table II).

Cannabinoid receptor expression in OA chondrocytes

To identify the expression of cannabinoid receptors CB1, CB2, PPARα and PPARγ in chondrocytes cultured in monolayer immunocytochemistry was used. Here we observed the expression of CB1, CB2, PPARα and γ [Fig. 5]. PPARα and γ expression was localised to the cytoplasm and nucleus [Fig. 5].

Discussion

Cartilage degradation is a pathological feature of both OA and RA. The inflammatory cytokine IL-1 plays a key role in cartilage destruction and stimulates increased production of MMPs by chondrocytes, resulting in the breakdown of collagen and proteoglycan. Here, we have shown that treatment of articular chondrocytes from human OA cartilage with cannabionid WIN-55 reduces the gene and protein expression of MMP-3 and MMP-13 in the presence of IL-1β, suggesting that cannabinoids may have potential in terms of arthritis therapy. In addition we have shown that WIN-55 significantly reduces the gene expression of TIMP-1 and TIMP-2 to below basal levels.

In this study we have used both monolayer and a 3D culture system for culture of chondrocytes. Chondrocytes that have been isolated from articular cartilage dedifferentiate in monolayer culture changing their matrix synthesis, with a decrease in type II collagen and aggrecan, developing a fibroblast like phenotype and an increase in collagen type I. Cannabinoids may reduce the gene expression of MMP-3 and -13. Furthermore, in this study we have shown that WIN-55 treatment alone significantly reduced both TIMP-1 and TIMP-2 to below basal levels. We have also demonstrated that WIN-55 treatment significantly reduced TIMP-1 and TIMP-2 gene expression in grades 0, 2 and 3 cartilage. It is interesting to note that the reduction in TIMP-1 and TIMP-2 gene expression was greatest in grade 3 cartilage. This suggests that WIN-55 treatment may be more effective in treating advanced OA.

Table II

<table>
<thead>
<tr>
<th>Treatment (48 h)</th>
<th>MMP-3 (ng/alginate bead)</th>
<th>MMP-13 (pg/alginate bead)</th>
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<td>DMSO (0.1%)</td>
<td>1.4 (0.0659)</td>
<td>327.5 (1.6407)</td>
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<tr>
<td>IL-1β (10 ng/ml)</td>
<td>38.4*** (0.5902)</td>
<td>1,092.0* (16.8345)</td>
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<tr>
<td>WIN-55</td>
<td>1.4*** (0.0178)</td>
<td>219.4*** (1.2038)</td>
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<tr>
<td>WIN-55 (10 μM) +</td>
<td>2.0* (0.0051)</td>
<td>270.7 (3.8866)</td>
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Results are expressed as mean and 95% confidence interval; *$P < 0.05$, ***$P < 0.001$, compared to DMSO vehicle control and +++$P < 0.001$ compared to IL-1β treatment alone.
reversed with the key phenotypic features of chondrocytes being preserved when cultured in a 3D system such as alginate beads. In this study, chondrocytes were treated with IL-1β to mimic inflammatory processes in an in vitro model of OA.

Synthetic cannabinoids WIN-55 and HU-210 reduce IL-1α induced proteoglycan and collagen degradation in bovine nasal cartilage tissue suggesting a chondroprotective effect of these compounds. Here, we have shown a possible mechanism by which WIN-55 may prevent IL-1β induced ECM breakdown in OA cartilage tissue via preventing the expression of MMPs. We have demonstrated that chondrocytes from different grades of OA cartilage modulate MMP-3 and MMP-13 expression in response to WIN-55 with and without IL-1β stimulation. These findings together with others, suggest that cannabinoids may be of importance in the treatment of arthritis.

Psychoactive cannabinoid ajulemic acid (AJA) reduced MMP-1, MMP-3 and MMP-9 release from fibroblast like synovial cells stimulated with IL-1α and TNFα. In vivo, AJA has also been shown to reduce the severity of adjuvant-induced arthritis and other non-psychoactive cannabinoids, cannabidiol (CBD) and HU-320 reduced inflammation and joint damage in murine collagen-induced arthritis.

The effects of WIN-55 on articular chondrocytes did not appear to be influenced by the grade of the cartilage they were isolated from when cultured in monolayer. Chondrocytes cultured in monolayer express MMP-3 and MMP-13 at very low levels following WIN-55 treatment. However when comparing the effects of WIN-55 in monolayer to alginate bead culture, a greater inhibitory effect on MMP-3 and MMP-13 gene expression was seen in alginate bead cultured chondrocytes. Interestingly a biphasic expression pattern of MMP-3 and MMP-13 in response to WIN-55 was observed. MMP-3 was expressed in grade 0 and grade 3 cartilage derived chondrocytes and abolished in grade 2 cartilage.
chondrocytes and MMP-13 was abolished in chondrocytes from all grades of cartilage when cultured in alginate beads. These varying responses to WIN-55 treatment in alginate bead culture may indicate that the expression of MMPs may be differentially regulated depending on the grade and extent of cartilage degradation and the culture method utilised. Studies have shown that cartilage tissue derived from different OA grades or normal aged cartilage may influence the response of the chondrocytes to different treatments. Interestingly biphasic effects have also been seen with other cannabinoids namely Δ9THC.

During OA there is thought to be an imbalance between MMP and TIMP expression which in part contributes to cartilage breakdown. We have shown that WIN-55 inhibits expression of both destructive MMPs and protective TIMPs involved in the pathogenesis of OA, indicating that inhibition may occur via a signalling pathway, which regulates both at the transcription level. Human MMPs and TIMPs share a common activating protein 1 (AP-1) site in their promoters which regulates their transcription. WIN-55 may have a differential effect on AP-1 activation via peroxisome proliferator-activated receptors (PPARs). WIN-55 has been shown to activate AP-1 via PPARs. In addition AP-1 may be involved in the activation of interferon β (IFNβ). Production of IFNβ may result in reduced levels of MMPs and TIMPs. IFNγ, but not MMP-1, -3 and TIMP-1 in fibroblast-like synovial cells both with and without IL-1β stimulation and synovial tissue from patients with RA, treated with IFNβ, showed reduced levels of MMP-1 and TIMP-1. Furthermore IFNβ has been shown to have anti-inflammatory properties in the treatment of arthritis. Conversely PPARγ agonists have been shown to reduce IL-1β induced MMP-1 expression in human synovial fibroblasts via inhibiting DNA binding of AP-1. WIN-55 also binds to PPARγ so could also act in this way. The biological activities of cannabinoids and the signal transduction pathways they induce or inhibit need to be further investigated in OA. Since TIMP-1 and -2 are decreased by WIN-55 in human OA chondrocytes it is unclear whether there is a change in MMP and TIMP balance following cannabinoid treatment. However the inhibitory effect of WIN-55 on MMP-3 and -13 expression would indicate a possible role of cannabinoids in suppressing IL-1β induced ECM degradation by MMPs.

We have observed the expression of both classical cannabinoid receptors CB1 and CB2 in human chondrocytes from OA cartilage at passage 2. CB1 and CB2 have previously been shown to be expressed at similar levels both at the protein and RNA level in synovia of patients with OA and RA and their expression is thought to play a role in the pathology of joint disease. CB1 and CB2 have previously been shown to be expressed at similar levels both at the protein and RNA level in synovia of patients with OA and RA and their expression is thought to play a role in the pathology of joint disease.

In conclusion, in human OA chondrocytes, the synthetic cannabinoid WIN-55 inhibits the expression of matrix degrading enzymes MMP-3 and -13 and their inhibitors TIMP-1 and -2 in the presence or absence of IL-1β. This suggests a possible mechanism by which cannabinoids may act to prevent ECM breakdown in arthritis. Cannabinoids could provide a dual role in the treatment of arthritis as disease modifying agents in addition to having anti-inflammatory properties. Although there is increasing evidence to suggest that cannabinoids may be of therapeutic value in the treatment of arthritis, further studies into the receptors and signalling pathways involved in the actions of cannabinoids need to be investigated in order to elucidate their effects on catabolic and anabolic mediators in both normal and arthritic cartilage.

Authors contributions

SD participated in its design, performed all the laboratory work and analysis and co-wrote the manuscript. JMW helped to conceive the study, secure funding, contributed to its design and coordination, participated in interpretation of data and co-wrote the manuscript. AC helped to conceive the study, secure funding, contributed to its design and coordination and co-wrote the manuscript. CLM helped to conceive the study, secure funding, contributed to its design and coordination, participated in interpretation of data and co-wrote the manuscript. RADB helped to conceive the study, secure funding, contributed to its design and coordination, participated in interpretation of data and co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

None declared.

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Supplementary data

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