Anti-inflammatory and pro-anabolic effects of 5-aminosalicylic acid on human inflammatory osteoarthritis models

Kaihu Li a,b, Yong Zhu a, Penghui Zhang b,c, Mauro Alini b, Sibylle Grad b, Zhen Li b,*

a Department of Orthopaedics, Xiangya Hospital of Central South University, Changsha, China
b AO Research Institute Davos, Davos, Switzerland
c Department of Orthopaedics, Xiangya Hospital of Central South University, Changsha, China

ARTICLE INFO

Keywords:
Chondrocyte pellet
Inflammatory model
Osteochondral explant
Osteoarthritis
Treatment
5-aminosalicylic acid

ABSTRACT

Background: Osteoarthritis (OA) is the most common degenerative joint disease, mainly affecting the elderly worldwide, for which the drug treatment remains a major challenge. Low-grade inflammation plays a pivotal role in OA onset and progression. Exploration of notable anti-inflammatory and disease-modifying drugs on human samples could facilitate the evaluation of therapeutic strategies for OA.

Methods: The anti-inflammatory drug 5-aminosalicylic acid (5-ASA) is a first-line drug for ulcerative colitis (UC), however no study has explored the effects of 5-ASA on articular chondrocytes. In this work, both in vitro (chondrocyte pellets) and ex vivo (osteochondral explants) human inflammatory OA models were applied to evaluate the effects of 5-ASA.

Results: In the inflammatory pellet model, 5-ASA remarkably downregulated the gene expression of interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) while upregulating proteoglycan 4 (PRG4) and cartilage oligomeric matrix protein (COMP) gene expression. Total glycosaminoglycan (GAG) synthesis by pellets was markedly increased in 5-ASA-treated groups compared with the inflammatory group. In conditioned medium, in vitro inflammatory explant model, 5-ASA mitigated signs of OA development by reducing inflammatory mediators and GAG loss.

Conclusions: These findings suggest that 5-ASA has anti-inflammatory and pro-anabolic effects on human chondrocyte pellet and osteochondral explant inflammatory OA models.

The translational potential of this article: Disease-modifying OA drugs are an unmet clinical need for the treatment of OA. Our study explored and demonstrated the anti-inflammatory and protective effects of 5-ASA on in vitro and ex vivo human inflammatory OA models, showing its translational potential for OA treatment.

1. Introduction

Osteoarthritis (OA) is the most prevalent musculoskeletal degenerative disorder, causing reduced quality of life and increasing economic burden globally [1,2]. OA affects both small (like joints in hands) and large (like knee and hip joints) diarthrodial joints, manifesting recurrent joint pain, swelling, transient morning stiffness, and limited joint motion [1]. As a whole-joint disease, the pathology of OA involves cartilage degeneration, synovitis, and subchondral bone remodeling [1,3]. Among the underlying pathophysiological mechanisms, chronic low-grade inflammation plays a pivotal role in OA onset and progression [3]. Although current widely-used drugs such as analgesics, steroids, and hyaluronic acid exhibit symptom-modifying and anti-inflammatory effects in basic and preclinical studies, none have shown notable clinical success in delaying or halting OA progression [4]. On one hand, these drugs do not possess disease-modifying functions. On the other hand, effective treatment is challenged by the complexity and heterogeneity of OA, in which extensive inflammation- and immunity-related signaling pathways are activated. Thus, there is a substantial clinical need to explore novel drugs which not only target multiple inflammatory pathways in OA development but also have disease-modifying effects for OA treatment.

5-aminosalicylic acid (5-ASA) (Fig. 1A) has been a first-line drug for over thirty years to treat ulcerative colitis (UC), a chronic inflammatory
bowel disease affecting the colon [5,6]. UC and OA share some similar pathogenic mechanisms such as activated NF-κB signaling pathway [5,7], increased Toll-like receptor 4 [5,8], tumor necrosis factor-α (TNF-α) [5,9], apoptosis [10,11], and oxidative stress [7,11]. 5-ASA’s mechanisms of action to induce UC remission include inhibition of cyclooxygenases, peroxisome proliferator activated receptor γ (PPARγ), NF-κB, and immunosuppressive effects [12,13]. Besides its extensive anti-inflammation efficacy, oral or topical 5-ASA also owns advantages of safety and little side effects after years of formulation development [6].

Hitherto, no studies have evaluated the effects of 5-ASA on OA. The current study aims to investigate the potential of 5-ASA for OA treatment. In vitro and ex vivo inflammatory OA models with human chondrocytes and osteochondral explants were used to test the effect of 5-ASA in inhibiting inflammation and mitigating OA progression.

2. Materials and methods

2.1. Reagents and 5-ASA preparation

All the reagents were purchased from Sigma unless otherwise specified. Dulbecco’s modified eagle medium high glucose (DMEM HG) was prepared by dissolving 13.38 g DMEM HG powder (Gibco), 3.7 g sodium bicarbonate, and 0.11 g sodium pyruvate in 1 L MilliQ water. After filtering through a 0.22 μm strainer, DMEM HG was stored at 4°C for further use. 5-ASA was first dissolved in DMEM. Then, the pH of 5-ASA in DMEM (around 6.0) was adjusted with 1 M NaOH to the same pH as the DMEM HG (around 8.0). After filtering through a 0.22 μm strainer, a storage concentration of 5-ASA at 40 mM was prepared for further use.

2.2. Human chondrocytes isolation and expansion

Cartilage fragments were dissected from osteoarthritic knee or hip joint cartilage of patients undergoing joint replacement operations with donors’ informed consent and ethical approval by cantonal ethical commission (KEK-ZH-NR: 2010-0444/0). Demographic characteristics of included donors were shown in Table 1. Macroscopically non-wear cartilage tissue with intact surface was minced into small pieces and digested in spinner flasks containing DMEM HG, 100 U/mL penicillin plus 100 μg/mL streptomycin (1% P/S, PAN™ Biotech), 10% fetal bovine serum (FBS, Seraplus), and 450 U/mL collagenase II (Worthington Biochemical Corporation) for 22 h at 37°C and 5% CO2. Dissociated cells were cultured in expansion medium, which consisted of DMEM HG, 1% P/S, 1% ITS+ Premix (ITS, Corning), 1% NEAA, and 50 μg/mL ascorbic acid (AA).

Table 1

| Purpose                  | No. | Gender | Age (years) | Kellgren-Lawrence grade | No. of explants |
|--------------------------|-----|--------|-------------|-------------------------|-----------------|
| Chondrocyte isolation    | 1   | Male   | 65          | 2                       | 2               |
|                          | 2   | Male   | 80          | 4                       | 4               |
|                          | 3   | Male   | 89          | 4                       | 4               |
|                          | 4   | Female | 70          | 3                       | 3               |
| Osteochondral explant    | 5   | Male   | 60          | 4                       | 4               |
|                          | 6   | Female | 58          | 3                       | 8               |
| isolation                | 7   | Male   | 74          | 4                       | 4               |

Figure 1. Experimental design and cell viability of chondrocytes treated with 5-ASA. A. Molecular structure of 5-ASA. B. Experimental design of in vitro (chondrocyte pellet) inflammatory OA model. C. Experimental design of ex vivo (osteochondral explant) inflammatory OA model. D. Cell viability of monolayer-cultured chondrocytes treated with various concentrations of 5-ASA (one way ANOVA, n = 12). Data were presented with mean ± SD. ***p < 0.001, ****p < 0.0001. B and C were made with Biorender.
2.4. Cell viability assay

Passage 2 chondrocytes were seeded in 96-well plates with 3000 cells per well overnight for cell attachment. The cells were treated in the absence (Control) or presence of 5-ASA at concentrations of 10 mM, 20 mM and 30 mM. After 24-h and 48-h incubation, the medium in each well was replaced with 100 µL fresh medium and 20 µL CellTitre-Blue Reagent (Promega). After 4-h incubation, fluorescence of each well was measured by microplate reader at excitation wavelength of 560 nm and emission wavelength of 590 nm. Background fluorescence caused by medium was determined by average readings of wells without cells. Cell viability (%) was normalized to the average value of the Control group after 24-h incubation.

2.5. Experimental design

For in vitro study (Fig. 1B), passage 3 cells were plated in 96-well V bottom plates (Thermo Scientific) by adding 0.25 million cells in each well and 0.25 mL chondropermissive medium, which comprised DMEM HG, 1% F/S, 1% ITS, 1% NEAA, 50 µg/mL AA, 10 ng/mL TGF-β1, 0.1 µM dexamethasone (Dex). The plates were then centrifuged at 400 G for 5 min to obtain chondrocyte micromass. After one-week culture, pellets were randomly divided into 4 groups and supplemented with medium as follows: 1) Control: chondropermissive medium; 2) OA: chondropermissive medium + 0.25 mL fresh medium and 0.25 mL chondrogenic medium, which comprised DMEM HG, 1% F/S, 1% ITS, 1% NEAA, 50 µg/mL AA, 10 ng/mL TGF-β1, 1 µM dexamethasone (Dex), 1% dexamethasone (Dex) and 2 µM T3 (T3) for 7 days. 3) 5-ASA: chondropermissive medium + 0.25 mL fresh medium and 0.25 mL 5-ASA; 4) 5-ASA 20 mM: chondropermissive medium + 0.25 mL fresh medium and 0.25 mL 5-ASA. Medium was changed every 2–3 days and collected on day 3, 6, 8, 11, and 14 for further analysis. Pellets were harvested on day 3 (short term), 8, and 14 (long term) for gene expression, biochemical, and histological analysis.

For the ex vivo study (Fig. 1C), explants after preculture were randomly divided into 4 groups as for the in vitro study. According to our previous study [15], 5 ng/mL IL-1β in combination with 5 ng/mL TNF-α were used to induce the inflammatory OA model. Medium was changed every 1–2 days and collected at every medium change, while explants were collected on day 7.

2.6. Gene expression analysis

Total RNA from pellets was isolated using TRI Reagent (Molecular Research Center, MRC). Two Pellets in 1 mL TRI Reagent were collected on day 7. Total RNA from pellets was isolated using TRI Reagent (Molecular Research Center, MRC). Two Pellets in 1 mL TRI Reagent were collected on day 7.

2.7. Glycosaminoglycan (GAG) measurement

After washing in PBS, pellets were digested in 0.5 mg/mL Proteinase K (Roche) overnight at 56 °C, then the enzyme was inactivated at 95 °C for 10 min, and the digest frozen at −20 °C for GAG and DNA measurement. GAG content in pellets and conditioned medium were measured as previously described [15] with 1,9-dimethyl-methylene blue (DMMB) as color reagent and chondroitin sulfate as standard. The total GAG synthesis per pellet (GAGtotal) during culture comprised GAG release into the medium (GAGmedium) and GAG content in the pellet (GAGcontent), namely GAGtotal = GAGmedium + GAGpellet.

2.8. DNA quantification

DNA content in pellets was measured with Bisbenzimide (Hoechst 33258). Calf Thymus DNA (Invitrogen) was used to obtain a standard curve with maximum concentration at 12.5 µg/mL. Samples and standards with volume of 40 µL in duplicates were pipetted into a 96-well white plate. Subsequently, 160 µL Hoechst 33258 dye solution (1 µg/mL) was added into each well and incubated for 20 min with protection from light. Fluorescence was read at excitation wavelength of 360 nm and emission wavelength of 465 nm by microplate reader.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The content of the inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) in medium was evaluated by ELISA (R&D Systems) according to the producer's instructions.

2.10. Nitric oxide assay

Nitric oxide (NO) in medium was measured by Griess Reagent System (Promega Corporation) according to the manufacturer's instructions.

2.11. Immunohistochemistry staining

Snap-frozen cryosections (thickness: 10 µm for pellets, 8 µm for explants) were used for immunohistochemistry (IHC) staining. Samples were fixed in 70% and 100% ethanol subsequently for 10 min each at room temperature, then air dried overnight. Sections for aggrecan (ACAN) immunostaining were pretreated in 10 mM DL-Dithiothreitol (DTT) reduction solution for 2 h at 37 °C and then 40 mM iodoacetamide alkylation solution for 1 h at 37 °C. After washing in deionized water to remove the cryocompound, all slides were incubated in 0.3% hydrogen peroxide (H2O2) for 30 min to remove endogenous peroxidase. Afterwards, 1.5 U/mL hyaluronic acid was used for antigen retrieval of collagen type II (COL2) at 37 °C for 30 min, while 0.25 U/mL

### Table 2

| Oligonucleotide primers and probes used for qPCR. |
|-----------------------------------------------|
| Gene | Primer & Probe | Sequence/Catalogue number |
|------|----------------|--------------------------|
| RPLP0 | Forward Primer (5′−3′) | TGG GCA AGA ACA CCA TGA TG |
|        | Reverse Primer (5′−3′) | CCG ATA TGA GGG AGC AGT TTC |
|        | Probe (5′FAM/3′TAMRA) | AGG GCA CCT GGA AAA CAA CCC AGC |
| COL2  | Forward Primer (5′−3′) | GGC AAT AGC AGG TTC AGC A |
|        | Reverse Primer (5′−3′) | GAT AAC AGT CCT GCC CCA CTT ACC |
|        | Probe (5′FAM/3′TAMRA) | CCT GAA GGA TGG CGT CAG CAC AAG A |
| COX-2 | Forward Primer (5′−3′) | TGT TTG GAG TGG GTT TCA GAA ATA |
|        | Reverse Primer (5′−3′) | GAA AAG TGG TCA ACG CAG GGA TTT TIG |
|        | Probe (5′FAM/3′TAMRA) | AGA A |
| ACAN  | Forward Primer (5′−3′) | AGT CCA GAC GCC TCC TGT ACT CA |
|        | Reverse Primer (5′−3′) | CCG GAA GTG GCG GTA ACA |
|        | Probe (5′FAM/3′TAMRA) | CCG GAA TGG AAA CTT GCA TCA GAA TCA ACT |
| PRG4  | Forward Primer (5′−3′) | Hs00981633.m1 |
| COMP  | Reverse Primer (5′−3′) | Hs00164359.m1 |
| IL-6  | Forward Primer (5′−3′) | Hs00174131.m1 |
| IL-8  | Reverse Primer (5′−3′) | Hs00174103.m1 |

Note: Primers and probes presented with sequences were self-designed, while others with catalogue numbers were from Applied Biosystems.
chondroitinase ACII was applied in ACAN epitope retrieval under the same conditions. After treatment with horse serum for 1 h, sections were incubated with primary antibodies against COL2 (DSHB, CIICI; 2 μg/mL), and ACAN (DSHB, 1C6; 5 μg/mL) for 30 min at room temperature, successively followed by binding with biotinylated horse anti-mouse secondary antibodies (Vector laboratories, BA-2001; 1:200) for 30 min, ABC-complex (Vector laboratories) for 30 min, and DAB (Vector laboratories) for 4 min. Slides incubated with PBS under the same conditions, instead of primary antibody, were as used as negative control (Neg). Nuclei were stained with Mayer’s hematoxylin for 30 s. The percentage of positive stained area in the whole section was quantified using Image J software [16]. Briefly, positive-stained area was automatically calculated by setting an optimal threshold conforming to its original figure, while the whole pellet area was circled manually. The percentage of positive stained area was obtained by dividing the positive stained area by the whole pellet area and then multiplying by 100.

2.12. Safranin O/Fast Green staining

Sections were fixed with the same method as described above for IHC staining. Slides were then stained with Weigert’s hematoxylin for 10 min, followed by 0.02% Fast Green for 6 min and 0.1% Safranin O for 12 min as previously described [15]. The percentage of Safranin O-stained area in pellet section or unstained area in the explant section was quantified using Image J software. For each pellet section, Safranin O-stained area was automatically calculated by setting an optimal threshold, while the whole pellet area was circled manually. The ratio of Safranin O-stained area to its whole pellet area was used for the calculation of percentage value. For explant sections, the percentage of unstained area was assessed as previously described [15].

2.13. Statistical analysis

The statistical analysis was performed using GraphPad Prism 8.4.0. Shapiro–Wilk test was first carried out to assess data normality in each group. One-way analysis of variance (ANOVA) was then performed for data with normal distribution, while Kruskal–Wallis test was used for non-normally distributed data. Statistical significance was defined with a p < 0.05.

Figure 2. 5-ASA regulated anabolism- and inflammation-related gene expression levels of chondrocytes in pellet OA model after 3-day treatment (COX-2, COMP, one-way ANOVA; IL-6, IL-8, ACAN, PRG4, COL2A1, Kruskal–Wallis test; n = 7). Data were presented as boxplot; center line, median; box limits, 25th to 75th percentiles; whiskers, min to max. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 3. 5-ASA inhibited the release of inflammatory markers in pellet OA model. Cumulative content of IL-6 (A), IL-8 (B) and Nitric oxide (NO) (C) in the conditioned medium (IL-6, Kruskal–Wallis test; IL-8, NO, one-way ANOVA; n = 4). Data were presented as mean ± SD. a*, p < 0.05 compared with Control group; b*, p < 0.05 compared with OA group; c*, p < 0.05 compared with 5-ASA 10 mM group; d*, p < 0.05 compared with 5-ASA 20 mM group.

Figure 4. 5-ASA promoted GAG synthesis after treatment for 14 days in pellet OA model. GAG content (A), DNA content (B), GAG/DNA (C) per pellet. D, E. Cumulative GAG content released in medium. F. Total GAG synthesis by pellets after 14-day treatment period. G. Percentage of GAG in medium to total GAG synthesis. Data were shown as mean ± SD. n = 3. Statistical analysis was performed using one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
3. Results

3.1. Cytotoxicity of 5-ASA on chondrocytes

Cell viability of chondrocytes under stimulation of different 5-ASA concentrations was evaluated by CellTiter-Blue Reagent. After 24-h incubation, 5-ASA showed no toxic effects on chondrocytes, while chondrocytes in 30 mM 5-ASA group presented a significant cell viability decrease in comparison to the cells without 5-ASA treatment after 48-h incubation (Fig. 1D). Thus, 5-ASA at concentrations of 10 mM and 20 mM were applied for further studies.

3.2. 5-ASA modulates inflammatory and anabolic gene expressions in pellet model

To evaluate the early effects of 5-ASA on OA pellets at transcriptional level, pellets were collected after 3-day (short term) treatment. As shown in Fig. 2, inflammatory cytokines induced an OA-like phenotype as indicated by gene expression results, featured by decreased anabolic genes (ACAN, COL2A1, PRG4, COMP) and increased inflammatory genes (IL-6, IL-8, COX-2) in OA group in comparison with control group. 5-ASA at 20 mM sharply reduced gene expressions of IL-6 and cyclooxygenase-2 (COX-2) compared with OA group. Extracellular matrix (ECM)-related genes proteoglycan 4 (PRG4) and cartilage oligomeric matrix protein (COMP) were significantly elevated upon 20 mM 5-ASA treatment. Although not significant, 20 mM 5-ASA showed a trend in down-regulation of IL-8 and up-regulation of ACAN and COL2A1 gene expression. Moreover, 10 mM 5-ASA presented similar regulation trends with weaker effects.

3.3. 5-ASA attenuates inflammation in conditioned medium in pellet model

Conditioned medium on day 3, 6, 8, 11, 14 (long term) was collected to analyze released inflammatory mediators. Compared with the control group, cytokines significantly enhanced the release of IL-6, IL-8 and NO into conditioned medium in OA group. Cumulative content of IL-8 and NO in OA group were both markedly decreased by 10 mM and 20 mM 5-ASA treatment compared with the OA group (Fig. 3B and C). Cumulative IL-6 amount showed a similar trend of reduction in 5-ASA-treated groups although not significantly different from the OA group (Fig. 3A).

3.4. 5-ASA promotes total GAG synthesis in pellet model

Pellets were collected on day 14 (long term) to assess total GAG synthesis after long-term treatment with 5-ASA. GAG content per pellet was remarkably reduced in OA group compared with control group (Fig. 4A), while DNA content was comparable among all the groups (Fig. 4B). Compared with the OA group, GAG/DNA per pellet was significantly upregulated in 10 mM 5-ASA-treated group and showed a trend of increase in 20 mM 5-ASA-treated group (Fig. 4C). Moreover,
cumulative GAG release in medium was restored in 20 mM 5-ASA-treated group (Fig. 4D and E). According to the formula GAG_{total} = GAG_{medium} + GAG_{content}, 5-ASA, especially at 20 mM, could promote total GAG synthesis under inflammatory conditions (Fig. 4F). However, the synthesized GAG in 20 mM 5-ASA group was mostly released into the conditioned medium (95.49%), but not maintained in the pellet (Fig. 4G).

3.5. 5-ASA mitigates matrix loss in pellet model

Histological staining was carried out to observe the extracellular matrix distribution in pellets. Proteoglycan (PG) staining by Safranin O suggested no evident differences among all groups after 3-day treatment (Fig. 5A). After 8 and 14 days of culture, the OA group showed reduced PG staining compared with the control group, whereas the percentage of
**3.6. 5-ASA inhibits cartilage inflammation and degeneration in explant model**

To explore the translational possibility of 5-ASA for clinical practice, our preclinical inflammatory OA model established with human osteochondral explants [15] was used to validate the effect of 5-ASA ex vivo. Compared with the control group, explants stimulated with IL-1β and TNF-α showed higher gene expression of IL-6 and IL-8, and lower gene expression of ACAN and COMP. Although not significantly different from the OA group, 5-ASA at 20 mM showed a trend of down-regulation on gene expression levels of IL-6 and IL-8. Upregulated ACAN and COMP gene expression compared with the OA group indicated pro-anabolic effects of 20 mM 5-ASA (Fig. 8). Furthermore, inflammation markers of IL-6 and NO in medium were reduced upon treatment with 5-ASA at 20 mM (Fig. 9A).

Under normal culture conditions proteoglycan depletion in the superficial cartilage zone was comparable in different donors included in our study (Supplementary Fig. 1). Histologically, the percentage of proteoglycan loss in cartilage induced by IL-1β and TNF-α was restored by 5-ASA at both 10 mM and 20 mM concentrations (Fig. 9B and C). Taken together, these findings suggest 5-ASA could inhibit inflammation and mitigate cartilage degeneration in the explant inflammatory OA model.

4. Discussion

With the formulation development and advent of mesalazine, 5-ASA has been a standard treatment for patients with UC for over thirty years [5,6,12]. 5-ASA exerts multifaceted anti-inflammation mechanisms of action in UC treatment. Since OA shares some common pathogenic changes with UC, we explored the effects of 5-ASA in inflammatory chondrocytes and cartilage explants. In most previous studies, only single IL-1β or TNF-α was used to induce inflammatory OA models. As both cytokines are critical mediators controlling cartilage degeneration [9], in this study the combination of IL-1β and TNF-α was applied to induce models closer to the native OA microenvironment [17,18]. Moreover, the use of human-derived chondrocytes and explants could facilitate the translation of 5-ASA into clinical practice.

### Figure 8. 5-ASA upregulated anabolic gene expression and downregulated inflammatory gene expression of chondrocytes in explant OA model after 7 days treatment (n = 3–4). Statistical analysis was performed using Kruskal-Wallis test. Data were presented as boxplot; center line, median; box limits 25th to 75th percentiles; whiskers, min to max. *p < 0.05.

|            | Con | OA | 10 | 20 | 5-ASA (mM) |
|------------|-----|----|----|----|------------|
| **IL-6**   |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |
| **IL-8**   |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |
| **ACAN**   |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |
| **PRG4**   |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |
| **COMP**   |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |
| **COL2A1** |     |    |    |    |            |
| mRNA fold change | 1×10^-1 | 1×10^0 | 1×10^0 | 1×10^0 | 1×10^0 |

PG-stained area in pellets was dramatically increased upon both 10 mM and 20 mM 5-ASA treatment (Fig. 5A, B, and 5C). IHC staining revealed that ACAN-stained area was restored in both 5-ASA-treated groups after 8 days of culture (Fig. 6A and B), while COL2-stained area was only enhanced in the 20 mM 5-ASA group (Fig. 7A and B). These morphology analyses demonstrate a protective role of 5-ASA at protein levels in the pellet inflammatory OA model.
For clinical practice, various anti-inflammatory drugs have been developed for OA treatment. Oral selective cyclooxygenase 2 inhibitors such as celecoxib are common agents, although they have been associated with considerable gastrointestinal and cardiovascular complications and show no disease modifying effects [1]. Intra-articular corticosteroids and hyaluronic acid could alleviate short-term joint pain presumably because of their anti-inflammatory actions; however, both lack disease modifying efficacy [1]. Emerging biologic agents targeting IL-1β, TNF, β-nerve growth factor (β-NGF) or inhibiting nitrogen oxide have repeatedly revealed disappointing results from clinical trials [19]. These results indicate that blocking only one cytokine may not be sufficient to counteract OA progression [19]. Besides IL-1β and TNF, other cytokines like IL-6 are also appealing targets which have been detected in osteoarthritic cartilage and synovial fluid [20]. In this preclinical study, 5-ASA inhibited multiple targets (COX-2, IL-6, IL-8, and NO) and enhanced the synthesis of several ECM markers (ACAN, COMP, PRG4, COL2) in both inflammatory pellet and explant models, demonstrating its broad and potent effects of anti-inflammation and pro-anabolism.

Under the extensive inflammatory cascades in chondrocytes induced by IL-1β in combination with TNF-α, 5-ASA showed a remarkable downregulation of inflammatory markers in pellet model. Though mean values were reduced in 5-ASA groups in comparison to the OA group, gene expression of IL-8 and cumulative release of IL-6 only presented a downregulation trend without statistical significance. Large inter-donor variations among the samples from clinical patients may contribute to statistically insignificant comparison. In the pellet model, the expression of matrix molecules like ACAN, PRG4 and COMP were all upregulated upon 5-ASA treatment, which was further supported by Safranin O staining and histological analysis of ACAN. In our study, the tested gene expression levels varied significantly between the groups after 3-day (short term) treatment, while Safranin O staining still showed no differences. On the other hand, mRNA usually represents a transient status, whereas ECM proteins resulted from their cumulative synthesis or breakdown during culture duration. These reasons might cause the discrepancy of ECM markers between mRNA and protein levels.

In our pellet study, GAG content in medium of the control group was much higher than that in the OA group, seemingly contrary to the fact that GAG levels in synovial fluid of OA patients parallel their cartilage degeneration [21]. Unlike authentic native cartilage tissue, chondrocyte pellets consist only of cells prone to form cartilaginous structure. With this purpose, the GAG anabolism in pellets ran at a relatively high level in the control group, while the matrix structure required to retain the synthesized GAG was still not established. Inflammation disrupted this balance predominantly by inhibiting GAG synthesis. Therefore, in the OA group less GAG was released into the medium. However, the ratio of GAG in medium to total GAG synthesis was still higher in the OA group than that in the control group, showing inflammation could impair GAG.
retention, possibly due to diminished collagen production. Total GAG synthesis, especially upon 20 mM 5-ASA treatment, was totally regained to the same level as the control group, suggesting 5-ASA could promote GAG production under inflammatory conditions.

Histological analysis also confirmed the effectiveness of 5-ASA in retaining ECM components. However, the ECM structure in 5-ASA-treated groups was still not comparable to the control group. In this research, we not only applied two classical cytokines simultaneously, but also with multiple repetitions every 2–3 days, leading to constant combined effects that may be stronger compared to previous in vitro and in vivo studies. Though some potential drugs may be missed out under these harsh inflammatory conditions during preclinical drug screening, our models should facilitate the selection of drugs with superior treatment effects and higher translation potential.

To further investigate its application in human native cartilage tissue, we evaluated the effects of 5-ASA on inflammatory osteochondral explants extracted from human hip joints. Under inflammatory conditions, inflammation- (IL-6, IL-8) and anabolism-related (ACAN, COMP) gene expression levels in cartilage showed an improved trend upon 5-ASA treatment. Moreover, 5-ASA decreased upregulated inflammation mediators (IL-6, NO) in medium and maintained proteoglycan staining in cartilage. However, the applicability of 5-ASA still needs to be further evaluated, since only 4 donors were included to date. Side effects of 5-ASA, a therapeutic active agent in sulfasalazine, in humans have been minimized with years of formulation development [6]. Moreover, as a clinically approved first-line drug for mild and moderate UC for years, wide applicability of 5-ASA in heterogeneous populations has been tested [5]. Oral administration of 5-ASA is quite common and suitable for patients with UC, whereas articular cartilage is an avascular and aneural tissue in a joint cavity filled with synovial fluid, which makes oral drugs hard to reach the diarthrodial joint with high concentration. Nevertheless, intra-articular drug injection can be a great alternative to deliver drugs which target cartilage, as is shown by widely-used intra-articular injected sodium hyaluronate and betamethasone for patients with joint disorders. Similarly, it might be preferable to apply 5-ASA by intra-articular injection, instead of oral administration, to increase its concentration in joints and reduce its side effects. Though half-life of the drug in the joint could be tested in an in vivo small animal study, the anatomy and biomechanics of cartilage in small animals (thickness 30 μm in mice, 100 μm in rats) are considered quite different from human's (thickness 2.2 mm) [22], sometimes making findings on effective doses not beneficial for clinical translation. Therefore, a cutting-edge controlled drug delivery system may be needed and should be helpful to retain a high concentration of 5-ASA in the joint if it shows a great efficacy [23].

There also exist some limitations in our study. The most effective concentration of 5-ASA varied depending on the assays or markers, which needs more careful consideration about the optimal 5-ASA dose for clinical translation. Though total GAG could be restored by 20 mM 5-ASA, the GAG retention could not, demonstrating its limitation in anti-catabolism, which may be compensated by combination drug therapy. Moreover, the underlying mechanism of action of 5-ASA should also be explored in future studies, as well as its retention and penetration in cartilage in vivo.

In summary, our findings reveal that 5-ASA has anti-inflammatory and pro-anabolic effects on human in vitro and ex vivo inflammatory OA models, which may be considered as a promising drug candidate for local OA treatment.

Funding
This study was funded by AO Foundation. Kaihu Li was funded by China Scholarship Council.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements
We acknowledge the kindness of Developmental Studies Hybridoma Bank (DSHB) who supplied the primary antibodies of ACAN and COL2 for immunohistochemistry staining.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.10.003.

References
[1] Martel-Pelletier J, Barr AJ, Cicuttini FM, Conaghan PG, Cooper C, Goldring MB, et al. Osteoarthritis. Nat Rev Dis Prim 2016;2:16072.
[2] Saffri S, Koliati AA, Smith E, Hill C, Bettapandi D, Mansournia MA, et al. Global, regional and national burden of osteoarthritis 1990–2017: a systematic analysis of the Global Burden of Disease Study 2017. Ann Rheum Dis 2020;79(6):819–28.
[3] Robinson WH, Leps CM, Wang Q, Raghu H, Mao R, Lindstrom TM, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol 2016;12(10):580–92.
[4] Mobasher A. The future of osteoarthritis therapeutics: targeted pharmacological therapy. Curr Rheumatol Rep 2013;15(10):364.
[5] Ungaro R, Mehandra S, Allen PB, Peyrin-Biroulet L, Colombel J-F. Ulcerative colitis. Lancet 2017;389(10080):1756–70.
[6] Le Berre C, Roda G, Nedeljkovic Protic M, Danese S, Peyrin-Biroulet L. Modern use of 5-aminosalicylic acid compounds for ulcerative colitis. Exp Opin Biol Ther 2020;20(4):633–78.
[7] Lepetit P, Papavassiliou KA, Papavassiliou AG. Redox and NF-κappab signaling in osteoarthritis. Free Radic Biol Med 2019;132:90–100.
[8] Gomez R, Villalvilla A, Largo R, Gualillo O, Herrero-Beaumont G. TLR4 signalling in inflammatory conditions during preclinical drug screening, our findings reveal that 5-ASA has anti-inflammatory and pro-anabolic effects on human in vitro and ex vivo inflammatory OA models, which may be considered as a promising drug candidate for local OA treatment.

Authorship
K.H. Li, S. Grad, and Z. Li contributed to the design of the study; K.H. Li, and Z. Li contributed to the acquisition, analysis, and interpretation of the data; K.H. Li drafted the manuscript; Y. Zhu, P.H. Zhang, M. Alini, S. Grad, and Z. Li revised the manuscript critically; and all the authors provided approval for publication of the content.

K. Li et al. Journal of Orthopaedic Translation 38 (2023) 106–116

115
[21] Kulkarni P, Deshpande S, Koppikar S, Patil S, Ingale D, Harsulkar A. Glycosaminoglycan measured from synovial fluid serves as a useful indicator for progression of Osteoarthritis and complements Kellgren-Lawrence Score. BBA Clin 2016;6:1–4.

[22] McCoy AM. Animal models of osteoarthritis: comparisons and key considerations. Vet Pathol 2015;52(5):803–18.

[23] Colella F, Garcia JP, Sorbona M, Lolli A, Antunes B, D’Atri D, et al. Drug delivery in intervertebral disc degeneration and osteoarthritis: selecting the optimal platform for the delivery of disease-modifying agents. J Contr Release 2020;328:985–99.