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by HPLC. SW1353 and primary human chondrocytes obtained after total knee replacement surgery from patients with osteoarthritis (OA) were cultured in DMEM with 10% FCS. Cytotoxicity of different CSE concentrations was examined using MTT and BrdU assays. Quantitative real-time RT-PCR (RT-qPCR) was used to evaluate the effects of CSE on mRNA levels *of interleukin-1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase* (*iNOS*), *cyclooxygenase-2* (*COX-2)* and *matrix metalloproteinase-3 (MMP-3)*. The nitric oxide (NO) production was measured using Griess assay. The gelatinolytic activity of MMPs was determined by zymography. Transient transfection and luciferase assay was performed to study CSE action on NF-kappaB signalling.

Results: Dose-response experiments revealed that up to 20 μg/ml CSE did not impair viability of chondrocytes. CSE treatments (5-20 μg/ml) significantly suppressed the up-regulation of proinflammatory cytokine mRNA (*IL-1β* and *TNF-α*) in response to IL-1β (10 ng/ml) stimulation in SW1353 and primary chondrocytes ($p<0.05$). At the same concentrations, CSE inhibited the IL-1β induced up-regulation of *MMP3* mRNA and gelatinolytic activity of MMPs in a dose-dependent manner. A significant inhibition of NO production and *iNOS* mRNA expression was also observed in IL-1β stimulated primary chondrocytes pretreated with CSE ($p<0.05$). Cotransfections of the COX-2 promoter luciferase reporter plasmid together with p50 and p65 expression vectors enhanced the COX-2 promoter activity 3.4-fold in SW1353 cells. This p50/p65-mediated transactivation of the COX-2 promoter was dose-dependently down-regulated to basal levels by CSE.

Conclusions: These results indicate that CSE possesses potent anti-inflammatory activity in osteoarthritic chondrocytes. In particular, its action might involve the inhibition of NO production via *iNOS* downregulation as well as the inhibition of p50/p65-mediated COX-2 promoter activation.

568

MODULATION OF CHONDROCYTE METABOLIC PATHWAYS BY NSAIDS AND THE CYCLOOXYGENASE-INHIBITING NITRIC OXIDE DONATOR (CINOD) NCX 429

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Purpose: Osteoarthritis (OA) progression is accompanied by a reduction of extracellular matrix and increased catabolism of collagen fibers and glycosaminoglycans in joints. Cyclooxygenase-inhibiting nitric oxide donators (CINODs) are novel anti-inflammatory compounds designed to provide balanced COX-1 and COX-2 inhibition while releasing nitric oxide (NO), an important modulator of vascular tone. In the cartilage, the role of NO is controversial, as it is a recognized marker of inflammation and a possible cause of chondrocyte loss, but also a potent immuno-modulating factor improving joint vascular perfusion. We investigated the effects of the CINOD NCX 429 and reference NSAIDs (naproxen and celecoxib) in stimulated adult rabbit chondrocytes, focusing on their catabolic and anabolic activities, as well as inflammatory parameters.

Methods: Primary cultures of adult rabbit (4-6 months) articular chondrocytes (RACs) were pre-incubated with test drugs (1-10 μM, 8 hours) before addition of IL-1β (10 ng/ml, 16 additional hours) in 5% O_2 conditions, to mimic joint limited perfusion. Levels of mRNA were determined by quantitative RT-PCR for assessing expression of extracellular matrix proteins (aggrecan and collagen type II) and matrix proteases [aggrecanases and matrix metalloproteases (MMPs)]. NO production was determined by measurement of nitrite/nitrate (NOx) using the Griess method. Type II collagen production was evaluated in the supernatant by Western blot analysis. To assess COX inhibition, prostaglandin E_2 (PGE_2) was measured by enzyme immunoassay in the supernatant of IL-1β stimulated chondrocytes (from 2 months-old rabbits) incubated with NCX 429 (1-100 μM).

Results: NCX 429 and naproxen did not show any impact on RAC viability. In IL-1β-stimulated RACs, NCX 429 induced a 2-fold increase in type II collagen expression, similarly to naproxen and celecoxib. NCX 429 stimulated type II collagen at both mRNA and protein levels. In addition, NCX 429 reduced IL-1β-stimulated expression of MMP-1 by ∼ 50% and aggrecanase-1 and 2 expression (by ∼ 50% and 40%, respectively), with similar efficacy to naproxen and celecoxib. Conversely, IL-1β-induced increase in MMP-3 and decrease in aggrecan expression were not prevented by NCX 429 or naproxen, whereas celecoxib inhibited MMP-3 expression by 40%. Interestingly, while naproxen and celecoxib increased IL-1β-stimulated NOx levels by ∼ 50 and 30%, respectively, NCX 429 decreased NOx levels (by ∼ 25%). Finally, NCX 429 inhibited IL-1β-induced PGE_2 biosynthesis in a concentration-dependent manner, indicating effective COX inhibition.

Conclusions: NCX 429, naproxen and celecoxib modulate IL-1β-induced expression of catabolic and anabolic markers in stimulated adult rabbit articular chondrocytes. Interestingly, the CINOD NCX 429 slightly reduces IL-1β-induced increase of endogenous NO synthesis, differently from the other NSAIDs, likely by inhibiting inducible nitric oxide synthase (iNOS) expression, as already reported for other CINODs. NO has been considered as potentially detrimental for chondrocytes; however, donation of low concentrations of NO from the CINOD NCX 429 does not seem to affect chondrocyte survival or to increase the expression of mediators involved in cartilage degeneration. CINODs may even inhibit endogenous overproduction of NO, and therefore may represent an interesting therapeutic approach for the treatment of osteoarthritis.

569

EFFECTS OF RIBONUCLEINATE COMPONENTS OF OSTEOCHONDRIN^R S OSTEOARTHRITIS THERAPY ON FORMATION AND ACTIVITY OF HUMAN OSTEOCLASTS *IN VITRO*

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Purpose: To establish if the ribonucleinate components of the osteoarthritis natural drug, Osteochondrin® S (OST) affect the bone-resorbing functions and gene expression in osteoclasts.

Methods: Human osteoclasts were generated *in vitro* from culture of RANKL-M-CSF stimulated peripheral blood mononuclear cells for 17d. Cells were treated with 23.5-587.2 ng/mL OST or 0.2-5 mg/mL RNA components of connective tissues or yeast (as used to make OST) and their effects on osteoclast formation were assessed using a Tartrate-Resistant Acid Phosphatase (TRAP) stain. Cell were also grown on dentine slices and used to determine the effects on osteoclast bone resorption was The pit formation on dentine was determined by scanning electron microscopy. In separate experiments, real time PCR was used to determine mRNA expression of principal enzymes or signalling molecules governing osteoclast functions.

Results: OST and its RNA components showed approximately equivalent concentration-dependent inhibition of TRAP stained osteoclasts and associated reduction in pit formation on dentine (Fig. 1A–F).

OST was, however, more potent in preventing bone resorption than the individual RNAs. mRNA expression of calcitonin receptor (CTR) was reduced

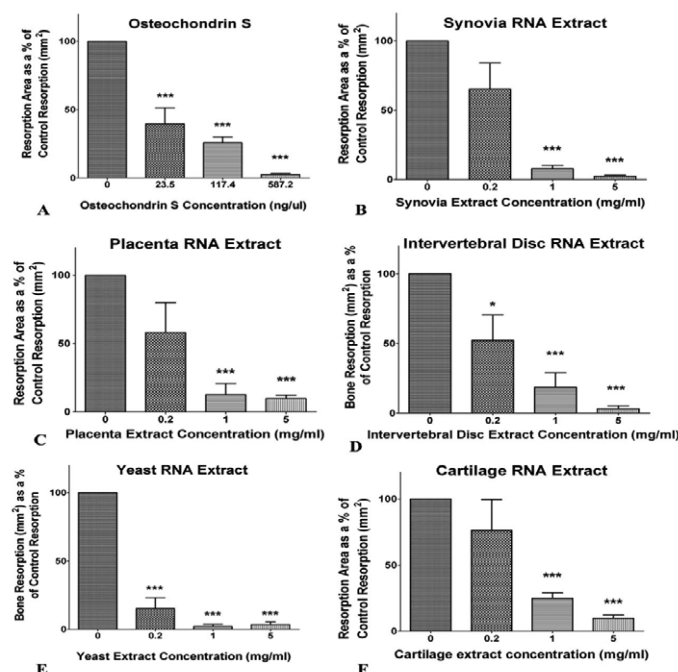

Figure 1A–F: Effects of OST and RNA components on osteoclast-mediated bone resorption. Statistical significance (***$p<0.001$; *$p<0.05$) - one-way ANOVA.

while Tumour Necrosis-α-like Weak inducer of Apoptosis (TWEAK) and interferon-β were increased over17d. No changes occurred in expression of mRNA for RANK, NFATc1, OSCAR or cathepsin-K.
Conclusions: The results show that bone resorption is inhibited by OST and its component RNAs in a concentrated-related manner. The inhibitory effects may possibly relate to events downstream of NFATc1 regulation.

570

THE CINOD NCX 429 EXERTS ANTI-INFLAMMATORY EFFECTS IN ISOLATED HUMAN MONOCYTES AND MACROPHAGES

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Purpose: Cyclooxygenase-inhibiting nitric oxide donators (CINODs) represent a new class of anti-inflammatory drugs designed to provide the anti-inflammatory effects of non steroidal anti-inflammatory drugs (NSAIDs) while donating nitric oxide (NO) with the aim of mitigating side-effects in the gastrointestinal and cardiovascular systems. CINODs exercise their analgesic and anti-inflammatory effects via cyclooxygenase (COX) inhibition, and exhibit an improved tolerability, via the protective action of nitric oxide (NO) on gastric mucosa and vascular function. We have assessed the effect of NCX 429, a naproxen-based CINOD, on human monocytes and monocyte-derived macrophages (MDM) isolated from healthy volunteers, in comparison with naproxen.
Methods: Human monocytes were isolated from heparinised venous blood of healthy donors by standard techniques in Hystopaque gradient centrifugation (400×*g*, 30 min, room temperature) and recovered by thin suction at the interface. MDM were prepared from monocytes, by culture (8-10 days) in RPMI 1640 medium containing 20% FBS.
Cells (1×10^6) were pre-treated for 1 h with NCX 429 or naproxen (1 nM - 100 μM) and then stimulated with 0.1 μM phorbol myristate acetate (PMA) for 24 h. Supernatant was collected at the end of the incubation for measurement of IL-6 release (ELISA).
To evaluate superoxide anion (O_2^-) production, human monocytes (1×10^6 cells/plate) were stimulated with PMA (1 μM, 30 min) in the absence or presence of NCX 429 or naproxen. O_2^- production was evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome C reduction and expressed as nmol cytochrome C reduced/10^6 cells/30 min. The MMP-9 activity was evaluated by gelatin zymography.
Results: NCX 429 in the range 1 nM - 100 μM inhibited PMA-induced IL-6 release in monocytes in a concentration dependent mode (IC_{50} = 870 nM); at the maximal concentration, the inhibition by NCX 429 reached 62±8%, significantly higher than the inhibition afforded by naproxen (36±4%, $p<0.05$, n=7). In MDM, 100 μM NCX 429 inhibited by 52±11% (n=5) PMA-induced IL-6 release, while naproxen had no effect. A stronger effect of NCX 429 was also observed by evaluating superoxide production in monocytes and MDM. At 1 μM, NCX 429 inhibited PMA-induced O_2^- production by about 24% in monocytes and 60% in MDM (n =6), whereas naproxen produced little or no effect. Moreover, NCX 429 reduced PMA-induced O_2^- production in monocytes isolated from patients affected by rheumatoid arthritis (n=3). Interestingly, NCX 429, but not naproxen, significantly inhibited matrix metalloproteinase (MMP)-9 activity in lipopolysaccharide (LPS)-challenged monocytes from healthy volunteers ($p<0.05$ *vs* LPS, n=3).
Conclusions: These results show additional anti-inflammatory effects of NCX 429 with respect to the reference NSAID, naproxen. CINODs, through NO donation, have been shown to have a favourable blood pressure and gastrointestinal profile relative to available NSAIDs in animal models; the present data suggest that NO donation from CINODs might also account for further anti-inflammatory effects beside the ones dependent on COX inhibition.

571

NAPROXCINOD, UNLIKE NAPROXEN, INHIBITS EXPRESSION OF iNOS IN ACTIVATED MACROPHAGES IN VITRO

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Purpose: Macrophages play a pivotal role in several inflammatory diseases and represent a valuable model for studying anti-inflammatory properties of new drugs. Activated macrophages produce and release numerous inflammatory mediators including cytokines, prostaglandins and reactive oxygen and nitrogen species. At physiologic concentrations, nitric oxide (NO) inhibits the expression and activity of pro-inflammatory mediators, whereas at high concentrations (i.e., during inflammation) it induces cytokine expression and tissue damage. Naproxcinod is the first-in-class cyclooxygenase inhibiting nitric oxide donator (CINOD), designed to exert effective anti-inflammatory actions through cyclooxygenase (COX) inhibition while releasing NO, with the aim of mitigating NSAIDs-associated unwanted effects. The purpose of this study was to characterize COX-inhibiting and NO-donating properties of the CINODs naproxcinod and NCX 429, and to investigate whether they modulate the expression of inducible nitric oxide synthase (iNOS) in activated macrophages differently from a reference NSAID, naproxen.
Methods: NO release was evaluated by assessing vascular tone on isolated rabbit aortic rings pre-contracted with methoxamine (3 μM) was recorded after incubation with vehicle or test compounds (0.01-100 μM) in the presence or absence of the soluble guanylyl cyclase (sCG) inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 10 μM).
To assess COX-1 inhibition, RAW 264.7 murine macrophages were incubated with test compounds (0.01-100 μM, 30 minutes) and stimulated with arachidonic acid (1 μM, 15 min). To assess COX-2 inhibition, aspirin (100 μM) pretreated cells were incubated with 1 μg/ml LPS and 10 ng/ml IFNγ.for 16 h to induce COX-2 expression and then treated as above. Esterases (1.67 U/sample) were added to accelerate naproxcinod metabolism. Supernatant was collected for enzymatic immuno-assay of PGE_2.
In different experiments, cells were incubated overnight with LPS (1 μg/ml) and IFNγ (10ng/ml) in the presence of vehicle or test compounds (0.1-100 μM) and nitrites quantified in the supernatant by the Griess reaction. Cells were lysed for Western blot and iNOS quantified by chemiluminescence.
Results: Naproxcinod and naproxen showed similar *in vitro* inhibition of COX-1 and COX-2 (COX-1 IC_{50}= 3.0±1.3 and 8.1±5.8 μM, respectively; COX-2 IC_{50}= 3.9±1.7 and 10.4±8.4 μM, respectively). Additionally, naproxcinod and NCX 429, but not naproxen, were able to induce relaxation of pre-contracted rabbit aortas (EC_{50}=5.5±1.7 and 6.2±1.6 μM, respectively), similarly to isosorbide mononitrate (EC_{50}=10.8±2.4 μM). The vasorelaxation was mediated by activation of the NO-signaling pathway, as prevented by the sGC inhibitor ODQ.
Interestingly, in activated RAW 264.7 macrophages, naproxcinod and NCX 429 (IC_{50}=0.8μM) but not naproxen inhibited LPS/IFNγ-mediated increase in nitrite concentrations. Consistently, naproxcinod reduced iNOS protein expression, whereas naproxen was ineffective.
Conclusions: The CINODs naproxcinod and NCX 429 inhibit expression of iNOS in activated macrophages *in vitro*, likely through a mechanism involving donation of NO. Therefore, in addition to the reported differentiated blood pressure and gastrointestinal profile over available NSAIDs, NO release from CINODs might confer additional anti-inflammatory properties.

572

KNEE OSTEOARTHRITIS: SAFETY AND EFFICACY OF CLODRONATE IV

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Purpose: To assess the clinical effects of intravenous courses of clodronate in established knee osteoarthritis (OA) resistant to common treatment with non-steroidal anti-inflammatory drugs or local corticosteroids
Methods: Subjects aged 40 to 80 years with knee OA, from moderate to severe, were enrolled. The patients were treated with a 10 days course of clodronate i.v. 300mg/die every 3-6 months for 1 year. Patients were followed-up every 3 months. At each visit, pain scores (100 mm visual analogue scale [VAS]), Lequesne index scores, NSAID intake, physician and patient global assessments scores were recorded. Adverse events (AEs) were recorded throughout the study.
Results: 122 patients (females 107, males 15; mean age 74.3±5.1, Kellgren-Lawrence grade II or III OA) were enrolled. Statistically significant reductions in VAS pain scores, Lequesne index scores and NSAID usage were reported at all time-points (baseline VAS 67.02±10.4, 1 month VAS 46.5±15.3, 6 months VAS 41.7±11.3, 12 months 37.2±4.3 $p<0.01$). No systemic, serious or severe side effects were observed.
Conclusions: This study supports the safety, tolerability and effectiveness of Clodronate in the treatment of symptomatic knee OA. Clodronate may also offer economic benefits due to a reduction in NSAID usage and the resultant reduction in management costs of NSAID related side-effects. This