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Different in vitro activity of flurbiprofen and its enantiomers on human articular cartilage

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Abstract

The 2-arylpropionic acid derivatives or 'profens' are an important group of non-steroidal anti-inflammatory drugs that have been used for the symptomatic treatment of various forms of arthritis. These compounds are chiral and the majority of them are still marketed as racemate although it is known that the (*S*)- form is the principal effective in the cyclooxygenase inhibition. However, recent findings suggest that certain pharmacological effect of 2-arylpropionic acids cannot be attributed exclusively to the (*S*)-(+) enantiomer. To obtain further insights into the pharmacological effect of profens, the present study investigated the influence of racemic and pure enantiomers of flurbiprofen on the production of nitric oxide and glycosaminoglycans, key molecules involved in cartilage destruction. The culture of human articular cartilage stimulated by interleukin-1β (IL-1β), which plays an important role in the degradation of cartilage, has been established, as a profit experimental model, for reproducing the mechanisms involved in the pathophysiology of arthritic diseases. Our results show that mainly (*S*)-(+)-flurbiprofen decreases, at therapeutically concentrations, the IL-1β induced cartilage destruction.

Keywords: (*S*)-(+)-Flurbiprofen; Arthritis; Cartilage; NO; GAGs

1. Introduction

The destruction of connective tissue matrix components in rheumatoid arthritis (RA) and osteoarthritis (OA) is a serious condition that causes the progressive destruction of joint. It is characterized by loss of articular cartilage and it has been suggested that interleukin-1β (IL-1β) plays the major role in the pathophysiology of these diseases [1–3]. In articular chondrocytes and synovial cells, IL-1β induces the net loss of cartilage proteoglycan by both stimulating the proteoglycan degradation and inhibiting the proteoglycan synthesis [3]. Moreover elevation of IL-1β induces the production of nitric oxide (NO) in fibroblasts and chondrocytes. NO is a free radical produced by three enzymes, called NO synthases [4–6]. Two NO synthases are constitutively expressed (eNOS and nNOS), the third one (iNOS) is expressed in response to cytokines or lipolysaccarides in smooth muscle cells, macrophages and hepatocytes as well as in chondrocytes and it could play a role in inflammatory and immunological host defence reactions [7,8]. NO has been shown to mediate some of the catabolic responses elicited by IL-1β in chondrocytes such as suppression of proteoglycan synthesis, induction of apoptosis, activation of collagenase gene expression and inhibition of cell proliferation [9–14].

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the widely used forms of therapy for OA. The traditional rationale for NSAIDs use is their ability to inhibit cyclooxygenase (COX) and hence suppress the inflammatory process and the pain [15,16]. Recently, it has been suggested that some NSAIDs might protect

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against cartilage and bone destruction in OA. Whereas other, such as indomethacin, might accelerate joint damage, possibly as a result of inhibitory effects on cartilage proteoglycan synthesis or by enhancing production of cartilage bone-destructive IL-1β or both [17–24]. There are several reports that certain NSAIDs may, in addition to inhibiting COX, modify cytokine production [25]. The inhibition of these cytokines by NSAIDs may be an alternative mechanism of action [26].

The 2-arylpropionic acid derivatives, or 'profens', constitute an important group of NSAIDs. Chemically they are weak acids and by virtue of a chiral carbon atom on the propionic acid side chain exist as enantiomer pairs, with (*S*)-isomers primarily responsible for inhibition of prostaglandin production and of inflammatory events [27–29]. The majority of these acids is still employed as racemate, this is the case for the flurbiprofen [(*R*,*S*)-2-(2-fluoro-4-biphenylyl)propanoic acid]. For our studies we have realised to separation of both the enantiomers resorting to two complementary enzymatic processes on the racemate as reported in Scheme 1.

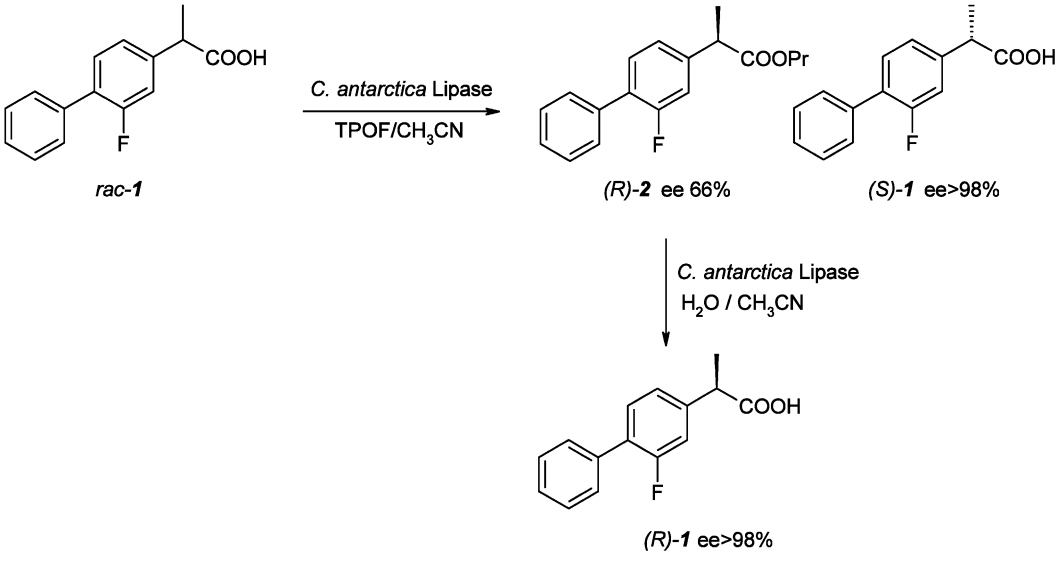
In this paper we have report the different properties of *rac*-, (*R*)- and (*S*)-flurbiprofen on the anti-inflammatory action, focusing our investigation on the production of NO and glycosaminoglycans (GAGs), key molecules involved in cartilage destruction. The enantiopure forms of flurbiprofen have been obtained through enantiomeric separation of the racemate by a modified biocatalysed procedure reported in literature [30,31]. The culture of human articular cartilage stimulated by IL-1β has been established as a profitable experimental model for reproducing the mechanisms involved in arthritic diseases [32,33].

2. Materials and methods

2.1. Preparation of enantiopure (*R*)- and (*S*)-flurbiprofen

To a solution of racemic flurbiprofen, (±)-**1** (1 g; 4.098 mmol) in CH_3CN (100 ml) containing tripropyl orthoformate (2.65 ml; 12.294 mmol), 10 g of lipase from *Candida antarctica* (Novozyme 435®) were added (Scheme 1). The mixture was incubated under shaking at 45 °C (300 rpm,) and the reaction progress was monitored by HPLC equipped with a chiral column (*S*,*S*)-Whelk-O1, 5 μm, 250 × 4 mm; hexane/2-propanol/acetic acid 98:2:0.5 as the eluent at a flow rate of 0.9 ml/min; for the enantiomers of **1** the selectivity factor (α) and the resolution factor (R_s) were 1.21 and 1.38, respectively). The ee value of flurbiprofen propyl ester **2** was calculated from $c = ee_s/(ee_s + ee_p)$ according to Ref. [34]. After 120 h the reaction was stopped and the enzyme removed by filtration. Removal of the solvent in vacuo left a residue that was portioned between hexane and $NaHCO_3$ solution. The organic phase, made anhydrous with Na_2SO_4, subjected to evaporation furnished 670 mg of (*R*)-**2** with 66% ee. Acidification of the aqueous phase, followed by extraction with hexane, gave 390 mg of *S*-**1** (yield, 39%) with ee >98%.

The enantioenriched ester (*R*)-**2** (500 mg) was dissolved in CH_3CN (50 ml) containing 500 μl of water and lipase from *C. antarctica* (1 g) was added. The mixture was incubated under shaking at 45 °C (300 rpm) until HPLC analysis indicated a conversion of 30% and ee value >98 for the (*R*)-acid produced. Workup as above of the reaction mixture furnished 150 mg of (*R*)-**1**. $[\alpha]_D = -42.7°$ (*c* 1 $CHCl_3$), Literature [30] $[\alpha]_D = +41.4°$ for (*S*)-**1** with ee >95%.



Scheme 1.

2.2. Drugs

The test drugs were dissolved in dimethyl sulfoxide, appropriately diluted in Dulbecco's Modify Medium (DMEM) and dispensed to the wells in 20 μl volumes to give the desired final concentrations.

2.3. Culture of human articular cartilage

Human articular cartilage was obtained at replacement surgery from patients with femoral neck fractures. Cartilage was reduced in fragments of small dimensions, and washed in 25 ml of Hank's balanced salt solution/antibiotic (penicillin 100 U/ml, streptomycin 100 μg/ml) and shaken vigorously for 30 s; such operation minimizes the risk of infection during the cartilage culture. The single fragments of cartilage were set in a sterile plate to 24 wells each containing 1 ml of DMEM phenol red free, 10% v/v foetal calf serum, 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 2.5 mg/ml of amphotericin B and it was kept in an incubator to 37 °C with 5% of CO_2 and 95% of air humidified for 24 h. After 24 h the culture media were removed and cartilage samples were treated. After 120 h the supernatants of cartilage culture were collected for different assays.

2.4. Determination of nitrite

Nitrite was determined by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 5% of chloridric acid) to 100 μl of samples [35]. The optical density at 570 nm was measured using a microtitrer plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite prepared in medium.

2.5. Determination of the GAGs

The level of GAGs was measured by spectrophotometry with a solution of 1,9-dimethylmethylene blue by the method of Farndale et al. [36,37].

The amount of GAGs was calculated from a standard curve obtained for shark chondroitin sulfate.

2.6. Statistical analysis

All the results presented are means ± SEM of three experiments performed on quadruplicate samples. The Student's *t*-test was used to evaluate the differences between the means of each group. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of rac-, (R)- and (S)-flurbiprofen on the IL-1β induced NO production

As showed in Fig. 1, NO production in cartilage under basal condition (control condition) is very low 16.70 ± 1.1 mainly due to activity of the (cNOS). When human articular cartilage was treated with IL-1β 10 ng/ml for 120 h a substantial increase in NO production to 62.79 ± 1.6 μM was observed probably due to up-regulation of iNOS.

Rac-, (*R*)- and (*S*)-flurbiprofen at 10 μM in combination with IL-1β reduced the production of nitrite induced to 37.61 ± 1.6, 39.43 ± 0.7, 34.93 ± 0.6 μM, respectively.

3.2. Effect of rac-, (R)- and (S)-flurbiprofen on the IL-1β induced GAGs release

In Fig. 2 the levels of GAGs are reported. The untreated control samples released, in the culture medium, a GAGs amount of 51.02 μg/ml. When the samples were treated with IL-1β at the dose of 10 ng/ml a release of 180 μg/ml was observed.

Rac-, (*R*)- and (*S*)-flurbiprofen at 10 μM in combination with IL-1β reduced the IL-1β induced GAGs release from matrix cartilage to 86.18 ± 1.3, 106.00 ± 2.5, 61.56 ± 1.9 μg/ml.

4. Discussion

Despite their therapeutic efficacy for pain relief in the treatment of OA, the role of NSAIDs in cartilage metabolism is still debatable. Several in vitro and in vivo studies have suggested that some NSAIDs promote degeneration of articular cartilage by inhibiting the biosynthesis of proteoglycans, which are crucial for maintaining the viscoelastic function of this tissue [38].

Flurbiprofen belongs to the 2-arylpropionic acid group of NSAIDs, and is available as racemate. In our work the esterification in organic solvent of *rac*-**1** catalysed by lipase from *C. antarctica*, using tripropyl orthoformate as nuclephyilic donor, furnished the enantiopure (*S*)-acid, (*S*)-**1**, as unreacted substrate and the enantioenriched (*R*)-ester, (*R*)-**2**. The corresponding hydrolysis of the (*R*)-**2** in the presence of the same lipase gave the enantiopure acid (*R*)-**1** (Scheme 1).

The (*R*)-flurbiprofen is before primarily considered to be the inactive isomer because it does not inhibit COX activity [28]. However, previous studies have revealed that it has antinociceptive and anti-tumor effects whereas (*S*)-isomer is primarily responsible for inhibition of prostaglandin production and of inflammatory events [29].

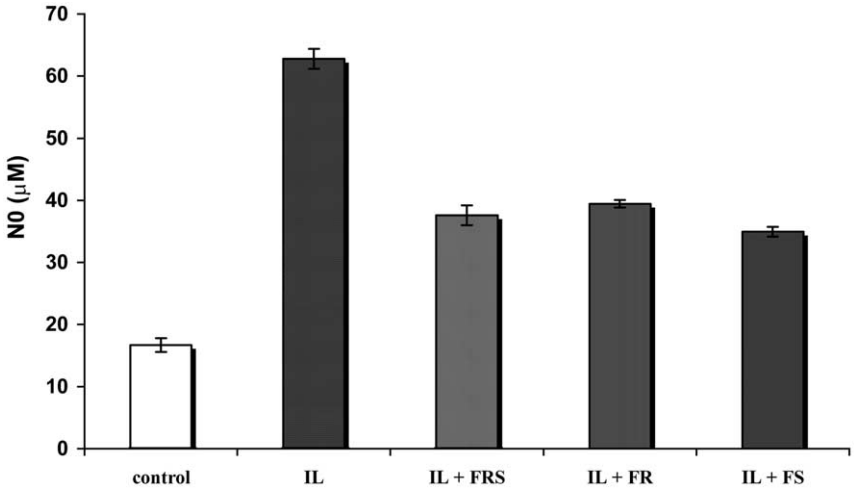


Fig. 1. NO production (means ± SEM) in the culture medium from articular chondrocytes 120 h after the addition of (10 μM) of *rac*, *S*, *R* flurbiprofen with IL-1β. Values are expressed as μM. *, significantly different from IL-1β treated samples ($P < 0.05$).

In this study we have investigated the effects of flurbiprofen as racemate and (*R*)- and (*S*)-enantioforms on production of NO and GAGs release in human articular cartilage stimulated with IL-1β. The potential role of NO in the pathophysiology of chronic inflammation has been extensively explored. Studies have demonstrated that sustained high NO levels can accelerate cartilage catabolism and reduce its anabolism [10–15] hence, interest in mechanism underlying the regulation of nitrite level as well as pharmacological approaches has substantially increased in recent years. Therefore we have examined the effect of *rac*-, (*R*)-, and (*S*)-flurbiprofen on the IL-1β induced production of NO and found that at concentration of 10 μM, which is within the range of plasma concentration, (*R*) and (*S*) enantiomers and racemate inhibited of 37.2, 44.3 and 40.10%, respectively with respect to IL-1β treated samples IL-1β induced nitrite accumulation in cartilage culture supernatants.

Next we examined the effect of *rac*-, (*R*)-, and (*S*)-flurbiprofen on the release of sulfated GAGs, which is likely to reflect proteoglycan release from chondrocytes. In general the erosion of cartilage in OA is initially caused by a decrease in the proteoglycan content, followed by destruction of collagen fibers. Therefore, we focused in the release proteoglycan in the matrix of

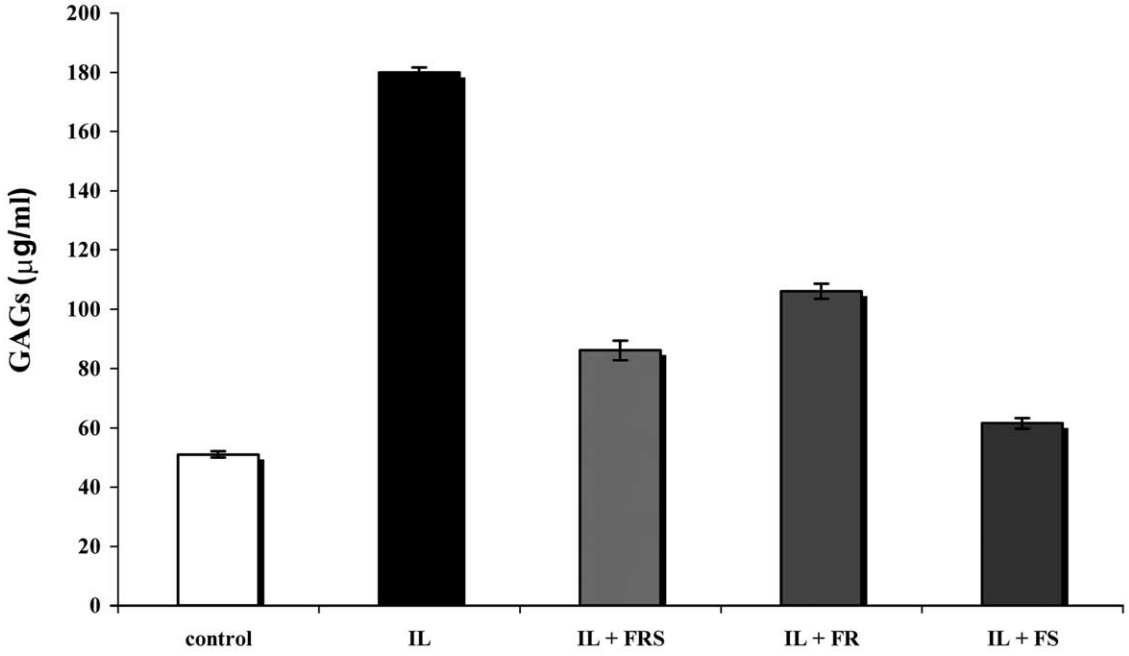


Fig. 2. GAGs release (means ± SEM) from articular chondrocytes 120 h after the addition of (10 μM) *rac*, *S*, *R* flurbiprofen, with IL-1β. Values are expressed as μg /ml. *, significantly different from IL-1β treated samples ($P < 0.05$).

cartilage culture a new type of chondroprotective properties.

It was demonstrated that flurbiprofen as (*R*) and (*S*) enantiomers and *rac* suppressed of 41.1, 65.5 and 52.12%, respectively the proteoglycan release induced by IL-1β. The reason why (*R*)- and mainly (*S*)-flurbiprofen effectively suppresses the release of proteoglycans is not understood. Probably might interfere with the production of metalloproteinases key enzymes for the pathological destruction of cartilage.

In conclusion, we have demonstrated that (*R*) and (*S*)-flurbiprofen suppress the interleukin-1 induced production of NO in a equipotent manner and that (*S*)-flurbiprofen reduces more breakdown of proteoglycans from human articular cartilage, respect to (*R*)-flurbiprofen.

It is very likely that the therapeutic effects of flurbiprofen in RA and/or OA are, at least in part, due to chondroprotective effects mainly evidenced for (*S*)-flurbiprofen, along the known inhibitory action of the prostaglandin E_2 production [39].

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