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A novel cyclo-oxygenase-2 inhibitor modulates catabolic and antiinflammatory mediators in osteoarthritis

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Abstract

ITB (6-(*p*-bromophenyl)amino-7-(*p*-chlorophenyl)indazolo[2′,3′:1,5]-1,2,4-triazolo[4,3-a]-1,3,5-benzotriazepine) is a novel inhibitor of cyclo-oxygenase-2 (COX-2) with antiinflammatory activity in animal models. In the present study, we investigated the effect of this compound on the production of catabolic or antiinflammatory mediators in osteoarthritis (OA) cartilage. In OA cartilage explants, ITB inhibited the production of prostaglandin E_2 (PGE_2), tumour necrosis factor-α (TNF-α) and matrix metalloproteinase-13 (MMP-13) in a concentration-dependent manner, whereas nitrite was partially reduced. On the contrary, ITB increased the production of interleukin (IL)-10 and the expression of heme oxygenase-1 (HO-1). ITB inhibited the production of catabolic mediators at concentrations able to increase IL-10 and HO-1 in OA cartilage, suggesting that this compound may be useful in the prevention of cartilage degradation.

Keywords: 6-(*p*-Bromophenyl)amino-7-(*p*-chlorophenyl)indazolo[2′,3′:1,5]-1,2,4-triazolo[4,3-a]-1,3,5-benzotriazepine; Cyclo-oxygenase-2; Heme oxygenase-1; Matrix metalloproteinase-13; Osteoarthritis; Interleukin-10

1. Introduction

Proinflammatory cytokines are mediators of inflammatory state and cartilage degradation in both rheumatoid arthritis and osteoarthritis (OA). In particular, interleukin (IL)-1β and tumour necrosis factor-α (TNF-α) activate chondrocytes to produce matrix-degrading factors and promote a catabolic condition [1]. These cytokines up-regulate inducible enzymes, such as nitric oxide synthase-2 (NOS-2) and cyclo-oxygenase-2 (COX-2) which is prominently expressed in the synovium, fibrocartilage of osteophytes, and in the blood vessels in the OA knee joint [2]. Heme oxygenase-1 (HO-1) is a stress-responsive protein with cytoprotective and antiinflammatory properties [3]. Nevertheless, the role of this enzyme in chronic inflammatory disorders has not been established. In this regard, we have shown in a recent report that HO-1 is expressed in human OA chondrocytes and can be modulated by cytokines [4].

The conversion of arachidonic acid to prostaglandin-H_2 ($PG)H_2$ by COX enzymes is the rate-limiting reaction in the synthesis of prostanoids, which are responsible for normal physiologic functions and also for inflammation and pain. COX-1 is expressed constitutively in most tissues including the gastrointestinal tract, kidneys, and platelets, whereas COX-2 is expressed at low levels in normal tissue, but it is strongly induced by inflammatory mediators (reviewed in [5]). Recently, a variant of COX-1 (COX-3) has been cloned [6]. Selective COX-2 inhibitors are as effective as traditional nonsteroidal antiinflammatory drugs for the treatment of arthritis and pain, with a lower incidence of gastrointestinal side effects [7].

Abbreviations: COX-2, cyclo-oxygenase-2; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; HO-1, heme oxygenase-1; IL, interleukin; ITB, 6-(*p*-bromophenyl)amino-7-(*p*-chlorophenyl)indazolo[2′,3′:1,5]-1,2,4-triazolo[4,3-a]-1,3,5-benzotriazepine; MMP-13, matrix metalloproteinase-13; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOS-2, nitric oxide synthase-2; OA, osteoarthritis; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor-α

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ITB (6-(*p*-bromophenyl)amino-7-(*p*-chlorophenyl)indazolo[2′,3′:1,5]-1,2,4-triazolo[4,3-a]-1,3,5-benzotriazepine) is a novel inhibitor of COX-2 activity. The antiinflammatory effects of ITB were demonstrated in animal models where this compound inhibited PGE_2 levels in inflamed tissues but not in stomach, in contrast to dual COX-1/COX-2 inhibitors. ITB also partially inhibited NO production by decreasing NOS-2 protein expression [8]. Here we report the inhibitory effects of ITB on the production of inflammatory and catabolic mediators as well as the positive modulation by this agent of protective strategies in OA cartilage.

2. Materials and methods

2.1. Drugs

ITB was prepared as reported previously [8]. The selective COX-2 inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) was kindly provided by Merck (Rahway, NJ).

2.2. Cartilage samples

Cartilage specimens were obtained from nine patients with the diagnosis of advanced OA (seven females, two males, aged 70 ± 5 years, mean ± S.D.) undergoing total knee joint replacement. Full-thickness pieces of cartilage were removed from the femoral condyles. Cartilage cylinders (3 mm diameter) were dissected from the tissue using a biopsy needle. Explants were transferred to 96-well plates containing DMEM/Ham's F-12 (Sigma) supplemented with penicillin–streptomycin. This medium was changed 24 h later and explants were left unstimulated or stimulated with recombinant human IL-1β (100 U/mL) (Biocarta) in presence or absence of test compounds. In another series of experiments, explants were incubated with IL-10 (10 ng/mL). Supernatants at 48 h of culture time were collected, centrifuged and stored frozen at −70 °C until analysis. Cartilage explants were weighed, washed with phosphate-buffered saline (PBS), fixed in 10% buffered formalin for 24 h at 4 °C, embedded in paraffin and cross-sectioned (3 μm thick). Nitrite, as index of nitric oxide production, and PGE_2 levels were determined by a fluorometric method and by radioimmunoassay, respectively, as reported [4]. Levels of TNF-α and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA; R&D Systems). Levels of matrix metalloproteinase-13 (MMP-13) were determined by ELISA (Amersham Biosciences Europe), according to the manufacturer's specification.

2.3. Immunohistochemistry

After deparaffinization, the slides were treated for 10 min with 3% H_2O_2 to inhibit endogenous peroxidases

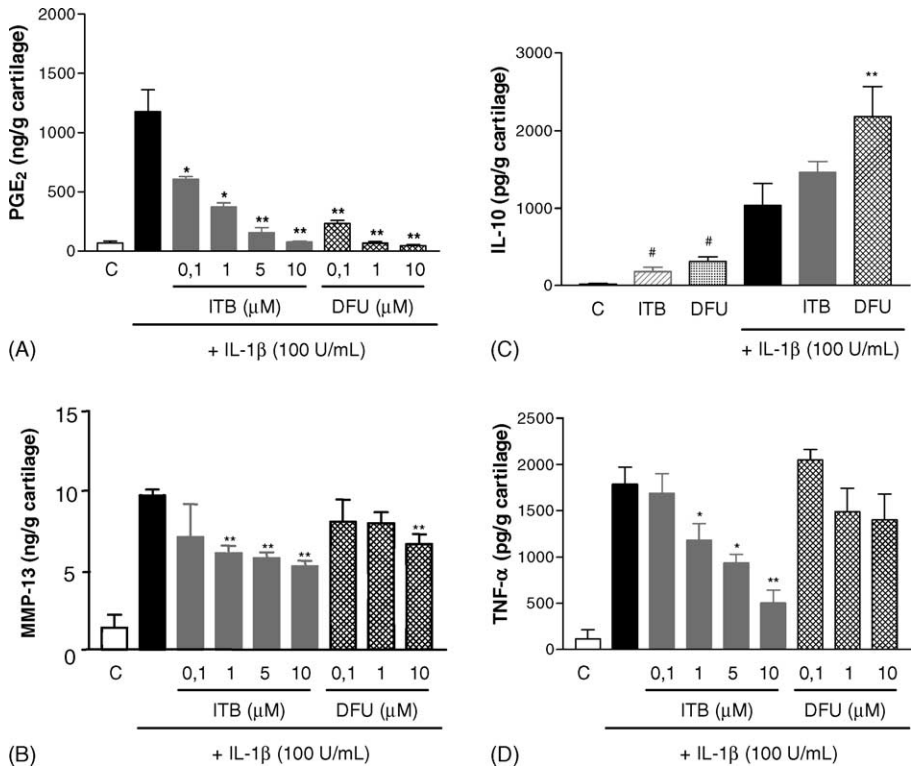

Fig. 1. Effect of test compounds (0.1–10 μM) on A, PGE_2, B, MMP-13, C, IL-10 and D, TNF-α production in cultured cartilage explants in the presence or absence of IL-1β (100 U/mL) for 48 h. In C, ITB and DFU were tested at 10 μM. Results are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to IL-1β-stimulated explants; $\#P < 0.01$ with respect to control (C); $n = 4$–6.

and incubated for 30 min with 5% normal goat serum in PTB buffer (0.02 M PBS, pH 7.0, 0.1% Tween-20, 2% bovine serum albumin and 0.1% sodium azide) to block non-specific binding. Anti-HO-1 polyclonal antibody (1/50) in PTB containing 1.5% normal goat serum was incubated for 60 min at 37 °C. After washing, a peroxidase-conjugated goat anti-rabbit IgG (1/200) in PTB containing 1.5% normal goat serum was incubated for 30 min at 37 °C. AEC substrate chromogen (DAKO) was used as substrate to detect positive cells. Counterstaining was performed with Mayer's hematoxylin (Sigma). Negative staining control experiments were performed according to the above-described protocol, with omission of the primary antibody.

2.4. Chondrocyte isolation and culture

Cartilage slices were removed from the femoral condyles and tibial plateaus and cut into small pieces. Chondrocytes were isolated by sequencial enzymatic digestion: 1 h with 0.1 mg/mL hyaluronidase followed by 18 h with 2 mg/mL collagenase (type IA) at 37 °C in DMEM/Ham's F-12 containing penicillin–streptomycin. The digested tissue was filtered through a 70 μm nylon mesh, washed and centrifuged. Cell viability was greater than 95% according to the Trypan blue exclusion test. All experiments were performed with chondrocytes in primary culture. The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [9] was used to assess the

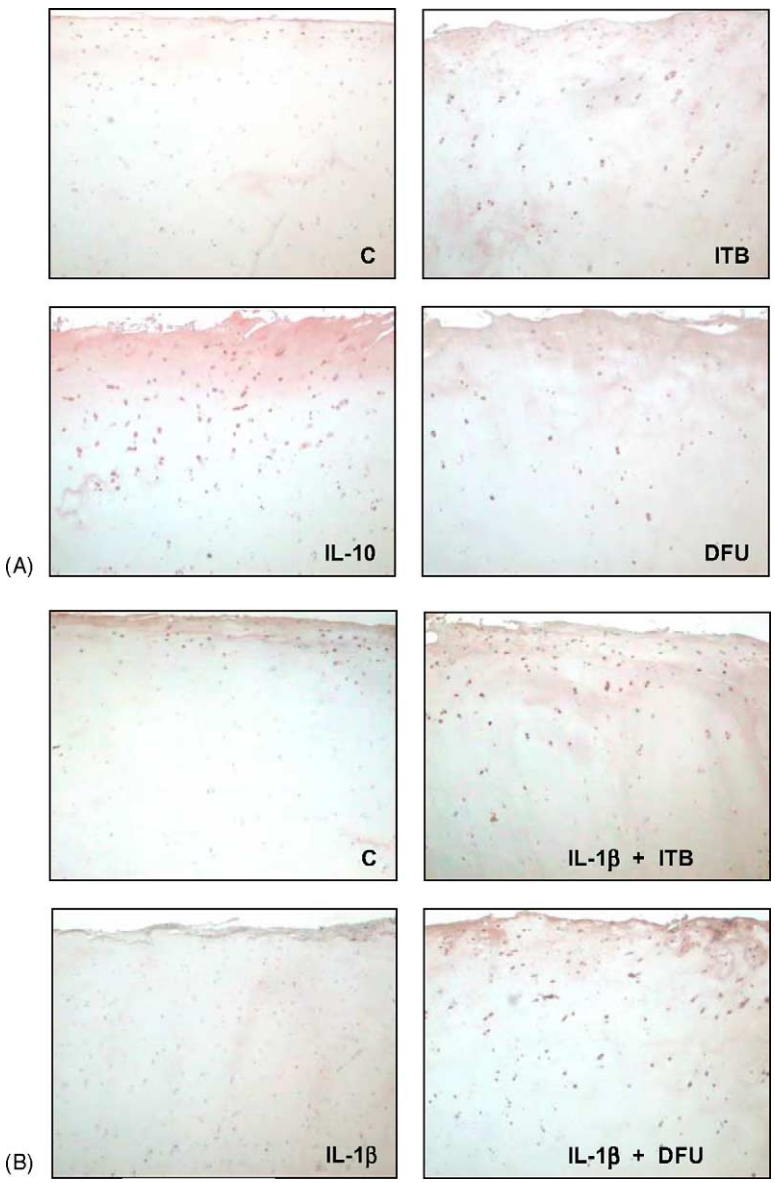

Fig. 2. Effect on HO-1 expression in cultured cartilage explants. A, non-stimulated explants. B, IL-1β-stimulated explants. ITB (10 μM) or DFU (10 μM) was incubated in the presence or absence of IL-1β (100 U/mL). IL-10 was used at 10 ng/mL. Incubations proceeded for 48 h. Representative photomicrographs of cartilage sections immunostained using a polyclonal anti-HO-1 antibody (magnification, 40×). Three experiments were performed with tissue from four different patients.

possible cytotoxic effect of test compounds on human chondrocytes. Chondrocytes were allowed to grow until nearly confluence and then incubated with IL-1β (100 U/mL) in the presence or absence of test compounds for 48 h. IL-10 (10 ng/mL) was also incubated for 48 h. Western blotting was performed as indicated [4].

2.5. Statistical analysis

The results are presented as mean ± S.E.M; *n* represents the number of patients. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test.

3. Results and discussion

First, we examined whether ITB was able to inhibit COX-2 induced by IL-1β in OA cartilage. Fig. 1A shows that ITB inhibited PGE_2 production in a concentration-dependent manner. We observed that ITB did not affect COX-2 expression in cartilage explants using immunohistochemistry (data not shown). These results confirm the effects of ITB on COX-2 reported in human monocytes, mouse macrophages and a cell-free system [8]. Interestingly, ITB also inhibited MMP-13 levels in supernatants (Fig. 1B), whereas DFU only was effective at the highest concentration (10 μM). ITB (10 μM) significantly reduced nitrite levels from 1412.5 ± 87.7 (IL-1β-stimulated group) to 1005.3 ± 115.3 ng/g cartilage ($P < 0.05$), although its effect was lower than that seen in murine cells [8]. In contrast, DFU did not affect nitrite production (1336.4 ± 138.6 ng/g cartilage, $P > 0.05$).

We also determined the effects of ITB on IL-10 and TNF-α production. It can be observed that both compounds at 10 μM increased basal IL-10 production, although statistical significance was only reached by DFU in the presence of IL-1β stimulation (Fig. 1C). shows that DFU failed to affect the levels of TNF-α, whereas the inhibitory effect of ITB was concentration-dependent. Incubations in the presence of PGE_2 (up to 400 ng/mL) did not prevent ITB effects, suggesting that they were not due to inhibition of PG production (data not shown). Additional mechanisms may be involved in the antiinflammatory effects of ITB, as we have shown in murine macrophages where this compound inhibited NOS-2 expression [8].

The expression of HO-1 was assessed by immunohistochemistry in sections of cartilage explants. HO-1 protein was detected in OA cartilage cultured in basal conditions and up-regulated after incubation with either ITB, DFU or IL-10 (Fig. 2A). As shown in Fig. 2B, IL-1β stimulation reduced HO-1 expression, whereas the presence of ITB or DFU clearly increased the levels of this protein. The effects of ITB and DFU on HO-1 expression were also assessed by Western blot using primary chondrocytes in culture. The concentration used was not toxic as assessed by the MTT test. In this system, IL-1β and IL-10 exerted opposite effects on HO-1 expression. Both COX-2 inhibitors increased HO-1 expression to a similar extent in cells cultured in basal conditions or stimulated with IL-1β (Fig. 3). An interesting finding of this study is the observation that ITB increased IL-10 production and HO-1 expression in human OA chondrocytes and cartilage, and these effects were reproduced by the selective COX-2 inhibitor DFU. This is in line with previous studies in murine cells showing that COX-2 inhibitors can potentiate the expression of HO-1 [10]. IL-10 inhibits the expression of IL-1β and TNF-α [11,12] and can be a potential target for therapy in OA [13]. Interestingly, this antiinflammatory cytokine induces HO-1 in OA cartilage and chondrocytes. It is possible that HO-1 plays a role in the defense strategies against oxidative injury or proinflammatory cytokine production [3] and thus HO-1 induction could be part of the antiinflammatory mechanisms of IL-10 in OA.

It is also relevant that ITB inhibited the production of TNF-α and MMP-13. MMPs play a critical role in the pathogenesis of rheumatoid arthritis and OA. In particular, MMP-13 efficiently degrades type II collagen in OA cartilage [14]. Recently, it has also been reported the production of this enzyme by human osteoblasts and its possible participation in the process of bone loss in rheumatoid arthritis [15]. ITB inhibited the production of catabolic mediators at concentrations able to induce protective mechanisms in OA cartilage, suggesting that this compound may be useful in the prevention of cartilage degradation. These findings make ITB an attractive

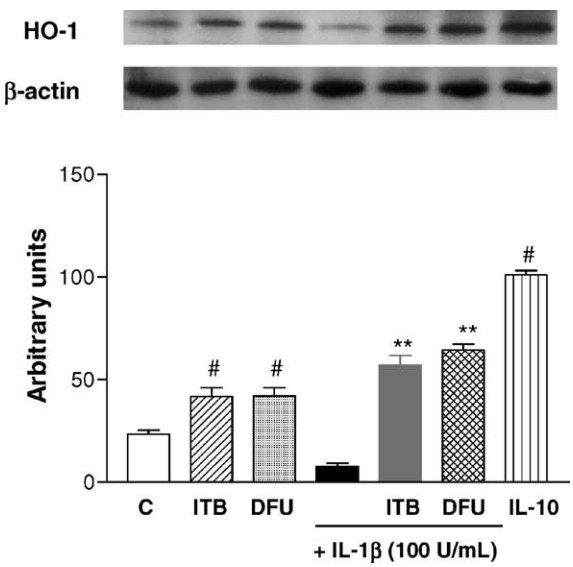

Fig. 3. Effect on HO-1 expression in primary chondrocytes. Cells were incubated with ITB (10 μM) or DFU (10 μM) in the presence or absence of IL-1β (100 U/mL). IL-10 was used at 10 ng/mL. Incubations proceeded for 48 h. Lower panel shows relative densitometric intensities. Results are presented as mean ± S.E.M. $^{**}P < 0.01$ with respect to IL-1β-stimulated explants; $\#P < 0.01$ with respect to control (C). Three experiments were performed with chondrocytes from three different patients.

molecule for further studies as a potential anti-arthritic agent.

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