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Thienopyrimidine derivatives prevent cartilage destruction in articular disease

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Abstract

The effects of a series of thienopyrimidine derivatives on the prevention of cartilage destruction in articular disease were investigated. Anti-degenerative activity was assayed on culture of nasal pig cartilage in the presence or in the absence of interleukin 1β (IL-1β). The amount of glycosaminoglycans (GAGs) and the production of nitric oxide (NO) in the culture medium were determined. Some thienopyrimidine derivatives, in the presence of IL-β, blocked the cartilage breakdown by inhibiting both the NO production and GAGs release in a dose-dependent manner.

Keywords: Cartilage; IL-1β; NO; GAGs; Thienopyrimidines

1. Introduction

Inflammation involves many chemical mediators and cell types. During the persistent pathological processes, involving chronic inflammation of the diarthroidal joints, enzyme factors, cytokines and inflammation promoting substances are released [1]. These cause cartilage destruction by an alteration of articular chondrocyte metabolism, leading to the cartilage matrix breakdown [2,3]. Although the precise mechanisms leading to cartilage breakdown are unknown, it was suggested that cytokines themselves induce inflammatory catabolism on the tissues involved in the pathological process [1]. Data showed that interleukin-1 (IL-1) interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) promote the cartilage destruction by chondrocytes activation leading to strong production of proteolytic enzymes [4–6]. The latter have the capability to destroy proteoglycans, components of cartilage matrix, tendons and protein of the bone. Other studies showed that IL-1β blockade prevents the cartilage breakdown [2,3]. The cytokines proinflammatory effects are due to the stimulation of the nitric oxide (NO) production.

NO is a multifunctional messenger molecule generated by a family of enzymes termed NO synthase (NOS). The inducible form of nitric oxide synthase (iNOS) releases high levels of NO in response to IL-1β and TNF-α [4,7]. NO inhibits the biosynthesis of matrix proteoglycans by inhibiting glycosaminoglycans (GAGs) synthesis and stimulates the chondrocyte production of proenzymes, that, following the conversion into active enzymes (the so-called matrix metalloproteinases—MMPS), cause cartilage breakdown [5–11].

Moreover, it has been demonstrated the paradoxical role of some commonly used non-steroidal anti-inflammatory drugs (NSAIDs), i.e. indomethacin, in the exacerbation of the IL-1β action on the cartilage destruction and their negative interference because of a downregulation of both the GAGs synthesis and the natural processes of chondroprotection [12–16].

The aim of current research is to develop anti-inflammatory molecules, capable of preventing cartilage destruction in articular disease. Several examples of NSAIDs having a thienopyrimidine system are reported to have interesting biological properties [17–19].

Recently, our interest in this field led us to prepare derivatives from 3-amino-2,3-dihydro-5,6-dimethyl-2-thioxo-thieno[2,3-*d*]pyrimidin-4(1*H*)-one and its hydrazinium salts [20].

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In the present work, we analysed the 'in vitro' effects of the same compounds on the metabolism of nasal pig cartilage treated or untreated with IL-1β, by measuring the amount of GAGs release and NO production into the culture media. Parallel experiments were also carried out in the presence of indomethacin.

2. Chemistry

2.1. Compounds

The acetic acid (**1A**), propanoic acid (**2A**) and phenyl acetic acid (**3A**) derivatives of 2-[(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)sulfanyl]-, the corresponding methyl esters (**1B**, **2B** and **3B**) and the 3-unsubstituted (**1C**), the 3-methyl (**2C**) and the 3-phenyl (**3C**) derivatives of 7,8-dimethyl-1*H*,9*H*-thieno[2′,3′:4,5]pyrimidino[2,1 - *b*][1,3,4]thiadiazine - 2,9-(3*H*)-dione were synthesised according to Scheme 1. The structures of above derivatives were supported by the results of elemental analysis as well as by the IR and ^{1}H NMR.

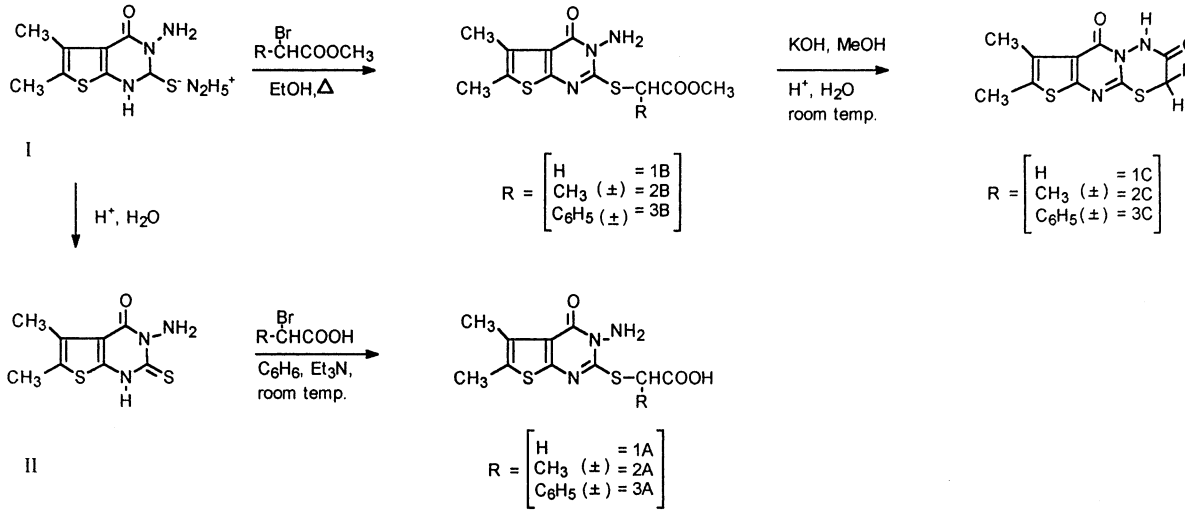
3. Pharmacological results

3.1. Effects on the NO release

In Fig. 1 the levels of NO are reported. The controls produced a very low amount of NO (18 ± 1.8 μM) mainly due to the activity of the constitutive NOS. A remarkable increase in the NO production up to 52 ± 2.1 μM was observed after the treatment of the samples with IL-1β at concentration of 10 ng/ml. The samples treated with indomethacin (10^{-5} M) showed a NO production close to that of the controls (20 ± 1.2 μM), whereas a significant decrease, relative to the IL-1β treated samples, was observed when indomethacin was combined with IL-1β ($P < 0.01$), but this response was significantly higher than in untreated control ($P < 0.01$). The compounds **1A**–**1B**–**1C** showed poor activity at the concentrations used in our study (1–10–100 μg/ml), with respect to the basal production. Compounds **1A**–**1B**–**1C** (at all the concentrations used) when combined with the IL-1β decreased the NO release compared to the samples treated with IL-1β. The effect was lower than that observed when the samples were treated with indomethacin and IL-1β. The compounds **2A**–**2B**–**2C**, did not give any significant effect on the NO release when used alone or in combination with IL-1β. The compounds **3A**–**3B**–**3C** in combination with IL-1β were very effective in reducing the production of NO whereas they were inactive when tested alone. This behaviour is quantitatively and qualitatively similar to that of indomethacin.

3.2. Effects on the GAGs release

In Fig. 2 the levels of GAGs are reported. The untreated control samples released, in the culture medium, a GAGs amount of 158 ± 1.6 μg/ml. When the samples were treated with IL-1β, at the dose of 10 ng/ml, a remarkable increase in the basal GAGs production was observed up to 280 ± 1.3 μg/ml. Similar value (165 ± 1.2 μg/ml) to that of the control group was observed for the samples treated with indomethacin only, whereas by combining the indomethacin with IL-1β significantly highest value was observed (250 ± 1.5 μg/ml). However, this latter value was lesser than



Scheme 1. Synthesis of the acetic acid, propanoic acid and phenyl acetic acid derivatives of 2-[(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-y)sulfanyl] (**1A**, **2A**, **3A**); corresponding methyl esters (**1B**, **2B**, **3B**); 3-unsubstituted, 3-methyl and the 3-phenyl derivatives of 7,8-dimethyl-1*H*,9*H*-thieno[2′,3′:4,5]pyrimidino[2,1-*b*][1,3,4]thiadiazine-2,9(3*H*)-dione (**1C**, **2C**, **3C**).

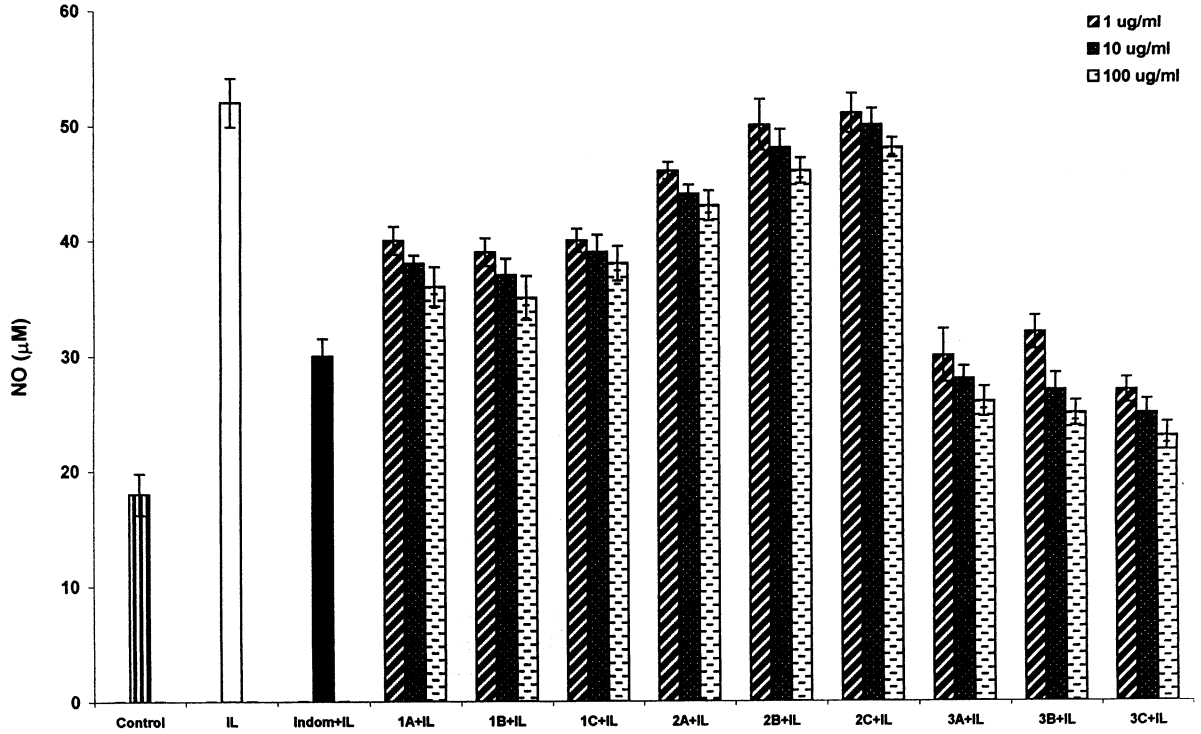


Fig. 1. NO production (means ± SEM) from porcine nasal cartilage in the culture medium 24 h after the addition of thienopyrimidine derivatives (1–10–100 μg/ml) and IL-1β or indomethacin and IL-1β. Values are expressed as μM.

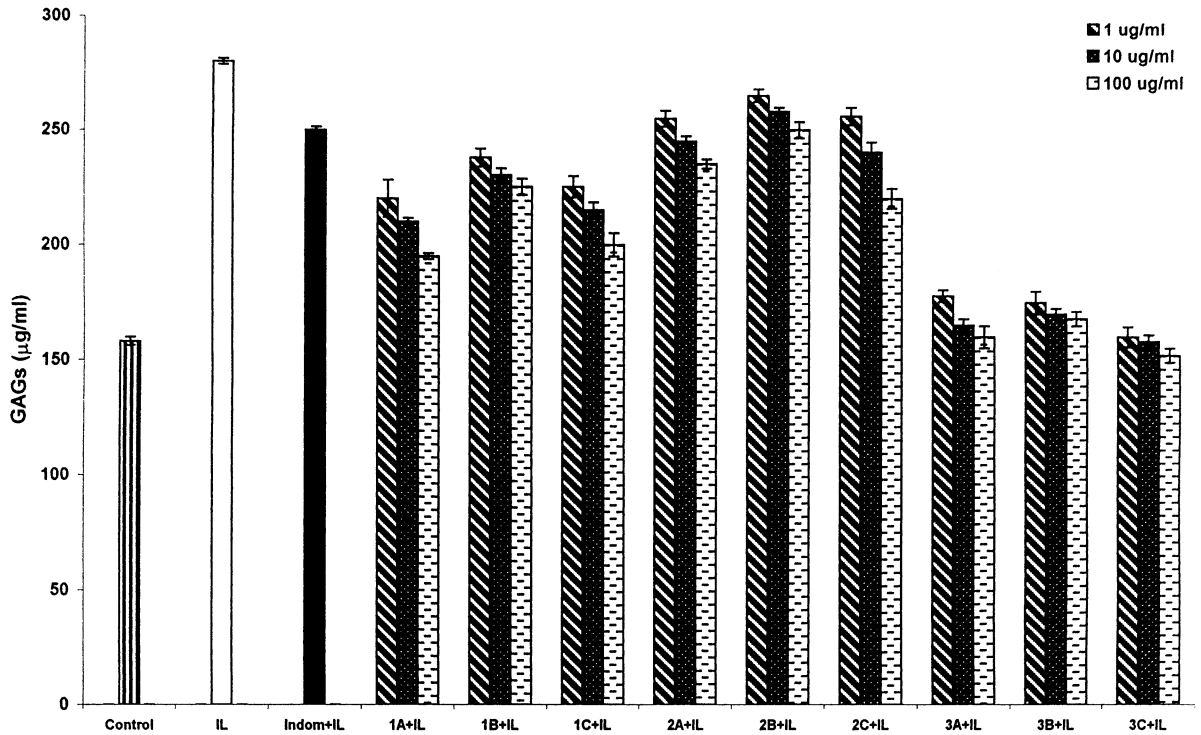


Fig. 2. GAGs release (means ± SEM) from porcine nasal cartilage in the culture medium 24 h after adding thienopyrimidine derivatives (1–10–100 μg/ml) and IL-1β or indomethacin and IL-1β. Values are expressed as μg/ml. Keys: Control = untreated samples; IL = IL-1β 10 ng/ml; Indom = indomethacin 10^{-5} M.

that observed when IL-1β alone was used. The samples treated with compounds **1A**–**1B**–**1C** produced a non-significant increase of the GAGs amount with respect to the untreated controls. A significant increase was observed when the samples were treated combining the compounds with IL-1β. The compounds **2A**–**2B**–**2C** were completely ineffective on GAGs release compared to the control samples. Compounds **2A**–**2B**–**2C** combined with IL-1β notably increased the GAGs release, but not relatively to IL-1β.

Compounds **3A**–**3B**–**3C** alone or combined with IL-1β only did not affect the basal release of GAGs. The observed values were very close to the control values.

4. Discussion

The potential role of NO in the pathophysiology of persistent pathological processes, involving chronic inflammation, has been extensively explored over the last few years [7–11]. Several in vitro and in vivo studies have demonstrated that this molecule and its products can accelerate cartilage catabolism and reduce its anabolism. It provides a valuable contribution to the development of new therapeutic strategies utilising compounds showing a selective inhibition of iNOS and to reduce the progression of some diseases. The GAGs serve as major constituents of the proteoglycan monomer providing hydrophilic negative charge within the cartilage extracellular matrix and, during inflammatory processes, are key molecules released from the cartilage matrix.

In previous papers, Russo et al. [19] and Santagati et al. (1995) have reported the synthesis and biological activities of derivatives of heterocycles containing the thieno[2,3-*d*]pyrimidine system. Several of these showed interesting analgesic and anti-inflammatory effects, as well as a very low acute toxicity and absence of ulcerogenic activity. As a continuation of research on derivatives of heterocycles containing the thieno[2,3-*d*]-pyrimidine system, we now investigated the effects on NO production and GAGs release to verify the activity of protection on pig nasal cartilage cultures treated with IL-1β. This cytokine was used to simulate the inflammatory process.

In the present study, we have demonstrated that, as reported, IL-1β increased the production of NO (nitrite) and GAGs release, and that, as expected, the thienopyrimidine derivatives showed no significant effect on the NO and GAGs production at the concentrations of 1–10–100 μg/ml, when used alone.

The same compounds showed a different behaviour when added to samples previously treated with IL-1β.

The compounds of the series **1** (thienopyrimidines without substituents on side chain) significantly decreased the NO production at all used concentrations with respect IL-1β treated cartilage, but produced a remarkable increase with respect to the untreated controls, lesser than that by indomethacin, in the GAGs production.

The compounds of the series **2** (thienopyrimidines with a methyl group in the side chain) were inactive in reducing the NO production of IL-1β treated cartilage. The same compounds increased the GAGs level in the supernatant, involving as indomethacin.

The compounds of the series **3**, bearing a phenyl group in the side chain, reduced the NO and GAGs release as triggered by the IL-1β. This clearly points out the essential role played by phenyl group in imparting pharmacological activity to this type of molecules containing the thieno[2,3-*d*]pyrimidine system. Probably, the presence of phenyl group makes the compounds of the series **3** more lipophilic than compounds of series **1** and **2**. Therefore, the better activity to prevent the IL-1β harmful effects on cartilage may be due to the better capacity to penetrate in the cartilage chondrocytes.

In conclusion, the thienopyrimidine derivatives, bearing a phenyl group on the side chain, are very promising to block the cartilage destruction during the inflammatory process as simulated in our experimental model.

5. Experimental protocol

5.1. Chemistry

Melting points (m.p.) are uncorrected and were determined in open capillary tubes in a Stuart Scientific SMP1 melting points apparatus. The IR spectra were recorded in a Perkin–Elmer 1600 Series FT-IR in potassium bromide disks. Elemental analyses for C, H, N, and S were obtained in an EA1108 elemental analyser Fisons-Carlo Erba instrument.

The ^{1}H NMR spectra were recorded at 200 MHz in a Varian Inova—Unity 200 spectrometer; chemical shift (δ) are reported in ppm from TMS as an internal standard. The purity of compounds was checked by TLC on Merck silica gel 60 F-254 plates. All commercial chemicals were purchased from Aldrich, Fluka, Merck and Carlo Erba and were used without further purification.

5.2. Preparations

Propanoic acid (**2A**) and phenylacetic acid (**3A**) derivatives of 2-[(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)sulfanyl]-, the corresponding methyl esters (**3B**) and the 3-methyl (**2C**) and the 3-phenyl (**3C**) derivatives of 7,8-dimethyl-1*H*,9*H*-thieno[2′,3′:4,5]pyrimidino[2,1 - *b*][1,3,4]thiadiazine - 2,9-

(3*H*)-dione were prepared according to the procedure reported in Santagati et al. [20].

2-[(3-Amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno-[2,3-*d*]pyrimidin-2-yl)sulfanyl] acetic acid (**1A**) was prepared according to the following procedure.

A mixture of 3-amino-2,3-dihydro-5,6-dimethyl-2-thioxothieno[2,3-*d*]pyrimidin-4(1*H*)-one (1.3 mmol), bromoacetic acid (97%, 1.4 mmol) and triethylamine (0.2 ml) in benzene (25 ml) was stirred at room temperature (r.t.) for 2 days. The resulting solid was collected and treated with 5% $NaHCO_3$. The aqueous solution, after filtration, was dropwise acidified with hydrochloric acid to pH 4–5, the resulting solid was collected, repeatedly washed with water and dried to give compound **1A** as a white amorphous powder.

Yield 72%; m.p. (dec.) 177–182 °C; IR (cm^{-1}): 3460 (OH), 3325 and 3270 (NH_2), 1730 and 1675 (C=O); ^1H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 3.84 (s, 2H, CH_2), 5.70 (s, 2H, NH_2), 12.70 (br s, 1H, OH); *Anal.* $C_{10}H_{11}N_3O_3S_2$ (C, H, N, S).

Methyl ester of 2-[(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)sulfanyl]-acetic acid (**1B**) was prepared according to the following procedure.

A mixture of hydrazinium (2.6 mmol) and methyl α-bromoacetate in ethanol (40 ml) was refluxed under stirring for 30 min and then stirred at r.t. for 1.5 h. The reaction mixture was poured into water (300 ml): the separated solid was collected, dried and recrystallised from ethanol to give a light yellow amorphous solid.

Yield 85%; m.p. 175 °C; IR (ν cm^{-1}): 3310 and 3205 (NH_2), 1735 and 1670 (C=O); ^1H NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 2.37 (s, 3H, CH_3), 3.65 (s, 3H, CH_3), 3.90 (s, 2H, CH_2), 5.82 (s, 2H, NH_2); *Anal.* $C_{11}H_{13}N_3O_3S_2$ (C, H, N, S).

Methyl ester of 2-[(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)sulfanyl]-propanoic acid (**2B**) was prepared according to the following procedure.

A mixture of hydrazinium (2.6 mmol) and methyl α-bromopropionate in ethanol (40 ml) was refluxed under stirring for 30 min and then stirred at r.t. for 1.5 h. The reaction mixture was poured into water (300 ml): the separated solid was collected, dried and recrystallised from ethanol to give a light yellow amorphous solid.

Yield 82%; m.p. 153 °C; IR (cm^{-1}): 3305 and 3195 (NH_2), 1738.0 and 1660 (C=O); ^1H NMR (DMSO-d_6): δ 1.50 (d, $J = 7.2$ Hz, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 3.67 (s, 3H, CH_3), 4.28 (q, $J = 7.2$ Hz, 1H, CH), 5.78 (s, 2H, NH_2); *Anal.* $C_{12}H_{15}N_3O_3S_2$ (C, H, N, S).

7,8-Dimethyl-1*H*,9*H*-thieno[2′,3′:4,5]pyrimido[2,1-*b*]-[1,3,4]thiadiazin-2,9-(3*H*)-dione (**1C**) was prepared according to the following procedure.

A suspension of ester (**1B**) (0.16 g) in a solution of NaOH (40 mg, 1.0 mmol) in methanol (10 ml) and water (2 ml) was stirred at r.t. for 24 h; the mixture was filtered and the filtrate poured into water (100 ml): by acidification of the resulting solution with HCl until pH 4–5 a white solid separated; this solid was collected, washed with water, dried and recrystallised from dioxane/water to give an amorphous white solid.

Yield 80%; m.p. 240–242 °C; IR (cm^{-1}): 3204 and 3115 (NH_2), 1680 (C=O); ^1H NMR (DMSO-d_6): δ 2.38 (s, 3H,CH_3), 2.39 (s, 3H CH_3), 3.86 (s, 3H CH_2), 11.74 (br s, 1H, NHCO); *Anal.* $C_{10}H_9N_3O_2S_2$ (C, H, N, S).

5.3. Biological activity

5.3.1. Nasal pig cartilage culture

Cartilage was obtained from a local abattoir, washed in Hank's balance salt solution containing penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively), sliced into small pieces (3–4 mm diameter), that were placed into 24 well plates, each containing 1 ml of Dulbecco' s modified Eagles medium phenol red free (DMEM, Sigma), glutamine (10 mM), penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively) and enriched with 10% heat inactivated foetal calf serum (30 min at 56 °C). After 24 h the media were removed and cartilage samples were treated as follows ($n = 4$ per group): (a) control medium, (b) compounds (1–10–100 μg/ml), (c) IL-1β (10 ng/ml), (d) compounds (1–10–100 μg/ml) combined with IL-1β (10 ng/ml), and (e) indomethacin (10^{-5} M). After 24 h the supernatants of cartilage culture were collected for different assays.

5.3.2. Determination of nitrite levels

Nitrite levels were determined in the culture media using the Griess reaction [21]. To single samples (100 μl), placed into 96 wells microplate, was added 100 μl of sulfanilamide (1% w/v). The plate was wrapped in aluminium foil, and shaken briefly. *N*-(1-Naphthyl)ethylenediamine (100 μl) was added to each sample, shaken briefly, wrapped with aluminium foil for 5 min.

The absorbance was measured at 570 nm using an automated plate reader, and finally the nitrite concentration determined from a sodium nitrite standard curve (0–120 μM).

5.3.3. Determination of glycosaminoglycans

GAGs were quantified using 1.9-dimethyl methylene blue (DMB) as previously reported [22,23]. A standard curve was used for the determination of GAG concentration (100–500 μg/ml). The standard GAG used is chondroitin sulfate C, derived from shark cartilage. The absorbance was measured at $\lambda = 535$ nm.

5.3.4. Statistical analysis

Each experiment was repeated at least three times in triplicate. The results were compared to control condi-

tions. Student's *t*-test and one-way ANOVA were used to calculate the significance of the differences between the means. All the statistical analyses were performed using the statistical software package SYSTAT.

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