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Synthesis and in vitro evaluation of 5-arylidene-3-hydroxyalkyl-2-phenylimino-4-thiazolidinones with antidegenerative activity on human chondrocyte cultures

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Abstract—5-Arylidene-3-hydroxyalkyl-2-phenylimino-4-thiazolidinones (**7**,**8**) were synthesized and evaluated for their antidegenerative activity on human chondrocyte cultures stimulated by IL-1β. This in vitro model has proven to be a useful experimental model to reproduce the mechanisms involved in arthritic diseases. The cell viability, the amount of GAGs, the production of NO and PGE_2 and the inhibition of MMP-3 were measured. Several thiazolidinones **7** and **8** exhibited the ability to block the production or action of the degenerative factors induced by IL-1β.
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1. Introduction

Osteoarthritis (OA) is one of the most common chronic diseases and its management represents an enormous cost to the health care system. OA is a multifunctional disease in which the principal target of the pathogenic process is the articular cartilage. Cartilage consists of a relatively small number of chondrocytes and many extracellular matrix (ECM) components, which comprise mainly collagen fibrils and aggrecan (a large aggregating proteoglycan). Chondrocytes synthesize and catabolize ECM macromolecules, while the matrix in turn functions to maintain the homeostasis of the cellular environment and the cartilage structure. In diseases such as OA the degradation of the ECM exceeds its synthesis, thereby resulting in a net decrease in the amount of cartilage matrix that depletes aggrecan. In addition, the disregulated synthesis of matrix components, such as proteoglycans (PGs) and collagen, plays a fundamental role.[1]

Moreover, the functional alteration of cartilage also results from the intervention of different cytokines, among which IL-1β proves to be significant. It induces high levels of proinflammatory mediators, such as prostaglandins E_2 (PGE_2) and nitric oxide (NO), and inhibits collagen and PG synthesis. IL-1β is a pivotal driving force in inducing and sustaining cartilage damage that usually leads to the loss of sulfated glycosaminoglycans (GAGs).[2] GAG release is a consequence of increased matrix protease activity, leading to the cleavage of collagen and PGs. Thus, GAGs can be considered an indicator of catabolic events in cartilage.

High levels of PGE_2 also mediate cartilage resorption by decreasing chondrocyte proliferation, enhancing matrix metalloproteinase (MMP) activity and inhibiting aggrecan synthesis in chondrocytes.[3] Several classes of MMPs have been implicated in tissue destruction: MMP-1 (interstitial collagenase), MMP-3 (stomelysin-1) and MMP-9 (gelatinase-B). These enzymes produced by chondrocytes have been reported to play a significant role in the destruction of the cartilage matrix in arthritic diseases. MMP-3 is particularly able to degrade matrix components including the core protein of the PGs.[4]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the long term treatment of inflammatory diseases, mainly for arthritic pain. They inhibit cyclooxygenase (COXs, also known as prostaglandin

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synthetase), which is the key enzyme in prostaglandin biosynthesis from arachidonic acid. Two isoforms of COX have been characterized: COX-1, a constitutive form that plays a cytoprotective role in the gastro-intestinal tract, and COX-2, a form generally induced in inflammatory sites.[5]

Recently, it has been suggested that a number of NSAIDs might accelerate joint damage, probably as a result of inhibitory effects on cartilage PG synthesis or by enhancing production of cartilage bone-destructive IL-1β. In addition, several reports have demonstrated that some NSAIDs may also modify cytokine production.[6]

Nowadays the main goal of research in this field is to find new anti-inflammatory drugs that are safer for long term use and that have a chondroprotective effect.

For a long time we have studied 4-thiazolidinone derivatives because of their interesting in vivo anti-inflammatory/analgesic properties along with a better gastrointestinal safety profile than those of known NSAIDs.[7] Moreover, some derivatives have been shown to be preferential inhibitors of COX-2 by suitable selectivity assays and supported by molecular modelling studies.[8]

As a part of an ongoing programme to design safer NSAIDs, 5-arylidene-2-phenylimino-4-thiazolidinones (**1**) proved to be interesting in vivo antiinflammatory compounds that reduce the carrageenan-induced paw and pleurisy oedema in rats. In the carrageenan-induced paw oedema assay, 5-(3-methoxybenzylidene)-2-phenylimino-3-propyl-4-thiazolidinone (**2**) was found to reach an inhibition level similar to that of indomethacin and had better activity profile than that of celecoxib (Fig. 1).[9]

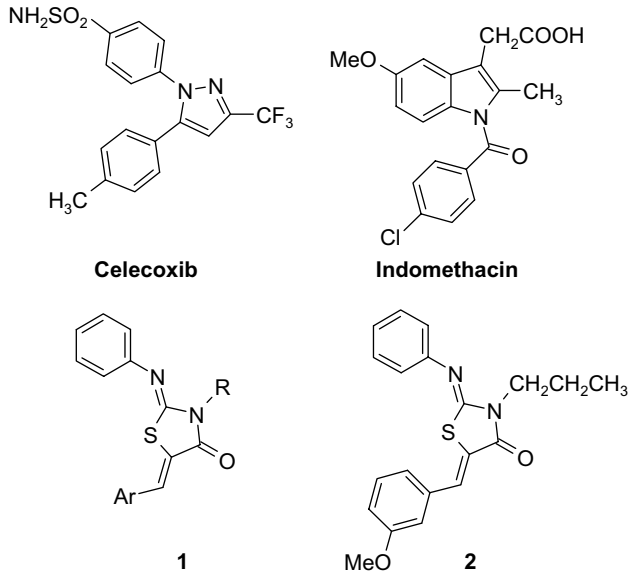


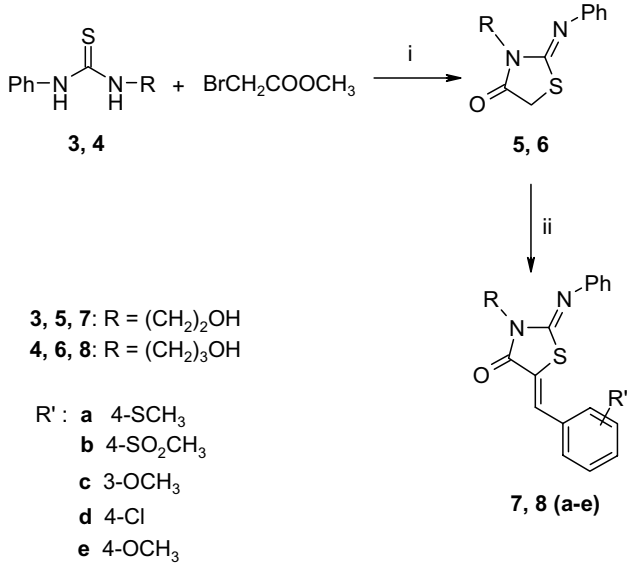
Figure 1.

In light of the above we here report the biological evaluation of a class of newly synthesized 5-arylidene-2-phenylimino-4-thiazolidinones, 3-hydroxyethyl (**7**) and 3-hydroxypropyl (**8**) substituted, in order to evaluate how is their in vitro anti-inflammatory and protective effect on inflammatory degenerative diseases as OA compared with **2**. A hydroxyl group on different alkyl chains in position 3 of the 4-thiazolidinone moiety was introduced in order to both increase their hydrophilicity and establish additional bonds with the biological target. The substituents on the 5-arylidene moiety were selected on the basis of the SARs which we previously acquired by means of in vivo anti-inflammatory assays.[9]

We decided to investigate particularly the chondroprotective effect of selected compounds **7** and **8** in cultures of human chondrocytes stimulated by IL-1β in an in vitro model that has proven to be a useful experimental model to reproduce the mechanisms involved in arthritic diseases.[10] The cell viability, the amount of GAGs, the production of NO and PGE_2 and the inhibition of MMP-3 were measured at different concentrations (10, 50 and 100 μg/mL). Indomethacin was used as reference drug.

2. Chemistry

Compounds **7**–**8** (**a**–**e**) were synthesized according to the synthetic pathway described in Scheme 1. The synthesis of 2-phenylimino-4-thiazolidinones **5**[11] and **6** was performed by condensation of *N*-hydroxyethyl- or *N*-hydroxypropyl-*N*′-phenylthiourea (**3**, **4**)[12,13] with methyl bromoacetate in the presence of triethylamine in ethanol at reflux for 24 h. The reaction provided only 3-hydroxyalkyl isomers **5** or **6**; however, when the reaction was carried out at room temperature in $CHCl_3$, both the phenylimino and hydroxyalkylimino isomers



Scheme 1. Reagents: (i) Et_3N, EtOH, Δ 24 h; (ii) ArCHO, piperidine, EtOH, Δ 24 h.

were isolated. In fact two possible reaction products could originate from the condensation of methyl bromoacetate with the sulfur atom of two different intermediate thiols generated from thioureas (**3**, **4**) by delocalization of the lone pairs of the two different nitrogens on the adjacent thiocarbonyl group. The type of solvent, the temperature and the thioureas substituents proved to be determinant for the reaction.[9]

5-Arylidene-2-phenylimino-3-hydroxyalkyl-4-thiazolidinones **7** and **8** were synthesized by the reaction in basic conditions of compounds **5** or **6** and appropriate aldehydes in refluxing ethanol for about 24 h. Compounds **7b** and **8b** were synthesized by oxidation with hydrogen peroxide in acetic acid of corresponding methylthio derivatives **7a** and **8a**, respectively.

The structure of all compounds was assessed by elemental analysis, ^{1}H and ^{13}C NMR. The resonances of alkyl protons allowed us to identify the isomer structure and by analogy of previously reported compounds. The NCH_2 protons appeared as a triplet resonating at chemical shift ranging between 3.93 ppm (**5**) and 3.65 ppm (**6**), due to the deshielding effect generated by the extended electronic delocalization of 3-N lone pair. In fact, the same protons of the alkylimino isomers resonated at lower chemical shifts.[9]

In ^{1}H NMR spectra of 5-arylidene-4-thiazolidinones derivatives (**7**, **8**), the NCH_2 protons appeared as a triplet resonating at chemical shift ranging between 4.01–3.90 ppm (**7**) and 3.68–3.66 ppm (**8**). Moreover, the absence of the signal of 5-CH_2 protons of starting compounds (**5**, **6**) together with the resonance of the methine hydrogen (7.72–7.93 ppm) agreed with the structures proposed above. Moreover, ^{13}C spectra showed a clear change in the splitting pattern of 5-C which resonated as a triplet in **5** and **6** and as a singlet in compounds **7** and **8**.

^{1}H NMR spectra and X-ray crystallography of a previously reported analogue allowed us to assign the *Z* configuration of the exocyclic C=C bond. In particular the methine proton resonance (7.73–7.77 ppm) became diagnostic; in fact it resonated at lower chemical shifts than *E* isomers owing to a greater deshielding effect of the adjacent C=O than that of 1-S.[14]

3. Results

3.1. Cell viability assay

MTT assay showed that all tested compounds did not reduce the ability of chondrocytes to metabolise tetrazolium salts, demonstrating that they did not interfere with cell viability at all tested concentrations (Fig. 2).

3.2. Determination of nitrite

Figure 3 shows NO production levels by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **2**, **7** and **8**, at the concentrations of 10, 50 and 100 μg/mL. It can be observed that when the tested compounds were combined with IL-1β this provided a significant reduction in NO release compared to the samples treated with IL-1β. In particular, compounds **7d**, **8d** and **8e** as well as compounds **7b** and **8a** at all tested concentrations proved to be the most efficacious derivatives as levels of NO production were lower than control samples (4.61 μM) and also indomethacin (7.56 μM). Such a result might be related to the inhibition of constitutive NO synthetase, an enzyme considered to be the mainly responsible for NO production under basal conditions.

3.3. Determination of GAGs

The total concentration of GAGs, an index of cartilage damage, in the medium of chondrocyte culture, is presented in Figure 4. It can be seen that the tested compounds combined with IL-1β showed a significant inhibition of IL-1β activity. At 100 μg/mL all the tested compounds by preventing the depletion of proteoglycan reported values similar to those of the untreated controls.

3.4. Determination of MMP-3

The results of the MMP-3 production are given in Figure 5. This study made it possible to demonstrate the ability of the tested compounds to suppress IL-1β-induced MMP-3 secretion in human articular chondrocytes. At 50 μg/mL all tested compounds significantly reduced MMP-3 secretion level reaching low values even compared with indomethacin. In particular, compounds **7a**, **7d** and **8b** achieved the greatest decrease of the MMP-3 secretion.

3.5. Determination of PGE_2

The cells treated with IL-1β (10 ng/mL) released significant levels of PGE_2. All tested compounds **2**, **7** and **8** reduced PGE_2 production without reaching the inhibition level of indomethacin (40.07 ng/mL). Compound **8b** was found to be the most active compound (53.22 ng/mL at 100 μg/mL) (Fig. 6).

4. Discussion

OA appears to be the result of an imbalance between the destructive and reparative/synthetic processes of the articular cartilage, in which some substances as free radicals (ROS and NO), inflammatory cytokines (e.g., IL-1 and TNF-α) and metalloproteinases (MMP) are produced in excess.[15,16] Therefore inhibitors of these mediators could represent a new type of chondroprotective drug for treating OA diseases. The above results showed that the protective activity of the tested compounds (**2**, **7** and **8**), assayed at different concentrations (10, 50 and 100 μg/mL), generally was dose dependent.

They strongly inhibited NO production induced by inflammatory IL-1β (Fig. 3). Both compounds **7** and **8** showed the same effect in reducing the IL-1β induced NO production independently of groups on 3-N, while

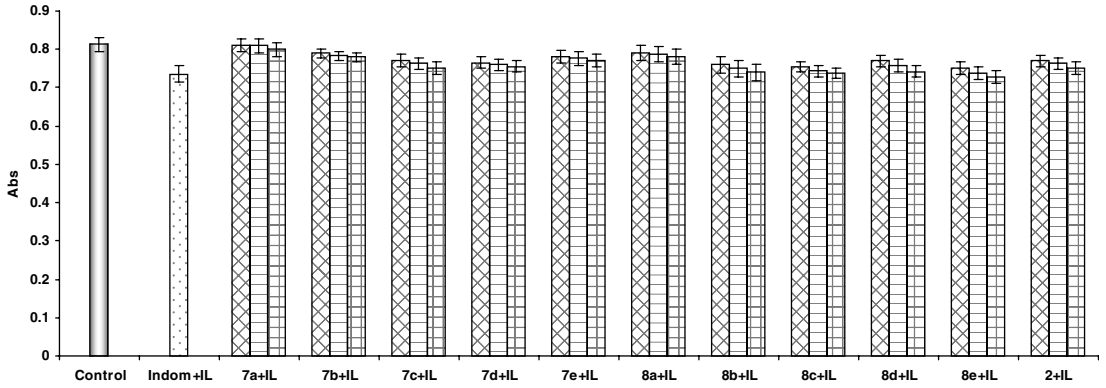


Figure 2. Cell viability (means ± SEM) in the culture medium by articular chondrocytes 120 h after the addition of compounds **2**, **7**–**8 (a–e)** at 10, 50 and 100 μg/mL, respectively. Values are expressed as Abs (absorbance).

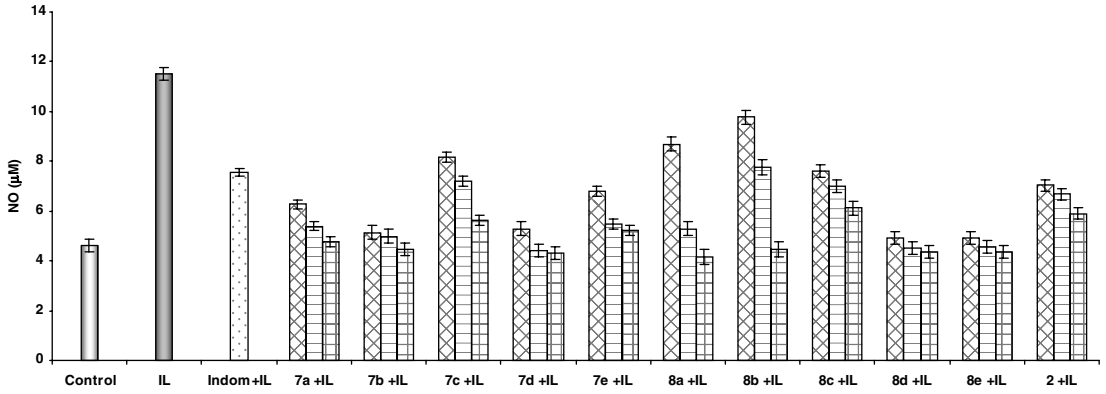


Figure 3. NO production (μM) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **2**, **7**–**8 a–e** at 10, 50 and 100 μg/mL, respectively. *Significantly different from IL-1β treated samples ($P < 0.005$).

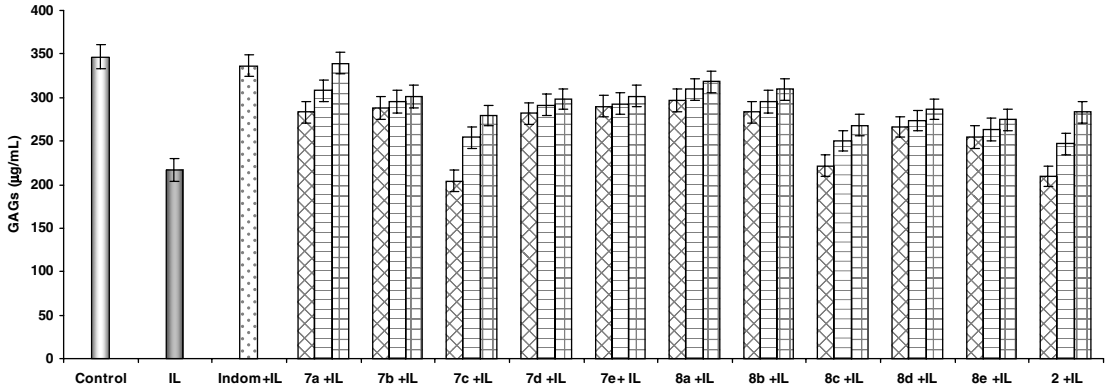


Figure 4. GAGs release (μg/mL) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **2**, **7**–**8 a–e** at 10, 50 and 100 μg/mL, respectively. *Significantly different from IL-1β treated samples ($P < 0.005$).

the substituents on 5-arylidene moiety appeared to influence the activity. Of the tested compound **8d** was the most active followed by **7d** showing that the chlorine atom was beneficial for activity; its replacement with OCH_3 was indifferent in the case of **8e** while it showed to be detrimental in the case of **7e**. The replacement of OCH_3 with SCH_3 resulted to be beneficial for compound **7a** but **8a** was less active at all tested concentrations. The oxidation of **7a** and **8a** to the corresponding sulfones (**7b**, **8b**) produced the same effect. The insertion of the OCH_3 group to the *meta* position (**7c**, **8c** and **2**) increased NO production levels thus having a detrimental effect on activity.

In the present study we observed that the tested 5-arylidene-2-phenylimino-4-thiazolidinones reduced the IL-1β-induced PG depletion as well as MMP-3 production (Figs. 4 and 5). In particular compounds **7** and **8** decreased the production of MMP-3, which ranged between 43.76 and 88.77 ng/mL at 100 and 50 μg/mL,

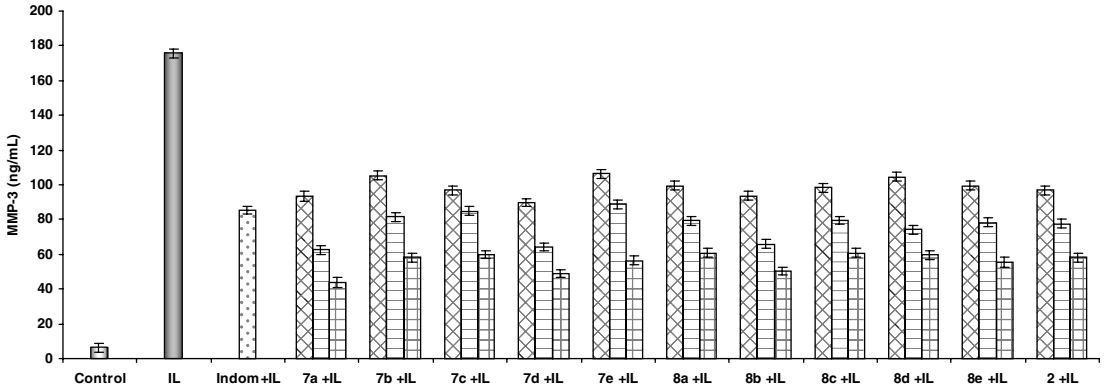


Figure 5. MMP-3 production (ng/mL) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **2**, **7**–**8 a–e** at 10, 50 and 100 μg/mL, respectively. *Significantly different from IL-1β treated samples ($P < 0.005$).

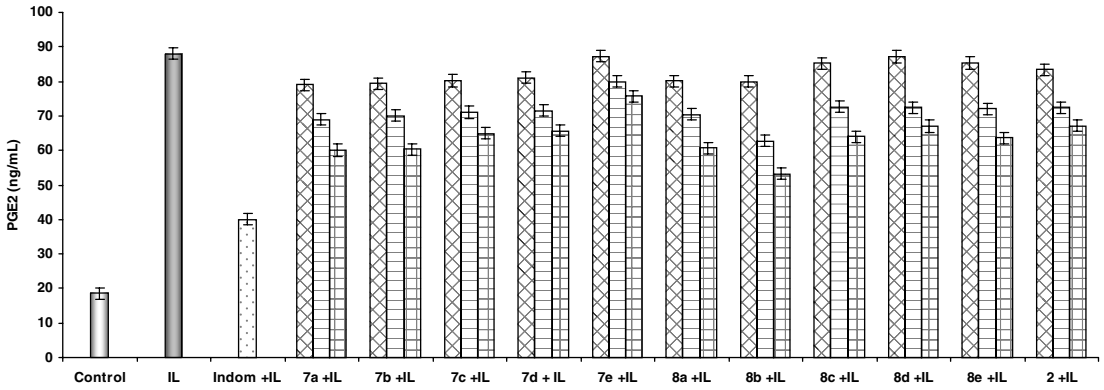


Figure 6. PGE_2 production (ng/mL) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **2**, **7**–**8 a–e** at 10, 50 and 100 μg/mL, respectively. *Significantly different from IL-1β treated samples ($P < 0.005$).

respectively, in comparison with indomethacin (62.64 ng/mL). In particular at 100 μg/mL **7b**, **7c**, **8a**, **8c**, **8d**, **8e** and **2** exhibited levels of MMP-3 production similar to that of indomethacin, whereas **7a**, **7d** and **8b** proved to be even more active. The ability to reduce MMP-3 enzyme appeared to be linked to the 5-arylidene-2-phenylimino-4-thiazolidinone skeleton, but to be independent of both N-3 and 5-arylidene substituents.

As regards the effect on GAGs, the treatment with all compounds reduced the catabolic response induced by proinflammatory cytokine. The best chondroprotective compound was **7a** (339.25 μg/mL at 100 μg/mL).

It is known that IL-1β mediates the inflammatory response via the stimulation of PGE_2 production. In our experiments, it can be observed that PGE_2 production was inhibited by all the tested thiazolidinones, without reaching the activity levels of indomethacin in any case. The most active compound was **8b**.

5. Conclusions

Overall, the experimental results here reported led us to suggest that the anti-inflammatory properties of 5-arylidene-2-phenylimino-4-thiazolidinones (**2**, **7**–**8**) are attributable to their ability to block the production or action of the degenerative factors induced by IL-1β. This may be beneficial in the treatment of arthritic disease.

Comparing the activity of compound **2** with **7** and **8** analogues indicates that the introduction of 3-hydroxyalkyl groups produces only minor effects on the antidegenerative activity of human chondrocytes. They generally show similar ability to block cartilage destruction except for the release of NO in the culture medium. In fact, compounds **7** and **8** significantly reduce NO production in comparison with **2**.

On the whole the antidegenerative activity of the selected thiazolidinones shows to be attributable to 5-arylidene-2-phenylimino-4-thiazolidinone system that appears promising for the development of novel attractive anti-inflammatory/antidegenerative agents by blocking cartilage destruction during the inflammatory process.

Further insights into the structural and biological properties of the compounds are in the process of being examined.

6. Experimental

6.1. Chemistry

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. TLC controls were carried out on precoated silica gel plates (F 254 Merck). Elemental analyses (C, H, N), determined by means of a C. Erba mod. 1106 elem. Analyzer, were within ± 0,4% of theory. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 magnetic resonance spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C). Chemical shifts are given in δ units (ppm) relative to internal standard Me_4Si and refer to DMSO-d_6 solutions. Coupling constants (J) are given in hertz (Hz). ^{13}C NMR spectra were determined by Attached Proton Test (APT) experiments and the resonances were always attributed by proton–carbon heteronuclear chemical shift correlation.

Unless stated otherwise, all materials were obtained from commercial suppliers and used without further purification.

6.2. Synthesis of 3-(3-hydroxypropyl)-2-phenylimino-4-thiazolidinone (6)

Methyl bromoacetate (2.29 g, 15 mmol) and triethylamine (2.02 g, 20 mmol) were added to a solution of *N*-3-hydroxypropyl-*N*′-phenyl-thiourea (**4**) (10 mmol) in ethanol (50 mL) and the mixture was refluxed under stirring for 24 h. The crude mixture was treated with ethyl ether until precipitation of triethylammonium bromide, which was eliminated by filtering. The filtrate was concentrated in vacuo and crystallized from ethanol. Yield 85%; mp 143–145 °C; ^{1}H NMR ($CDCl_3$): δ 1.92 (m, 2H, CH_2); 3.65 (t, 2H, NCH_2, J = 5.7 Hz); 3.86 (s, 2H, 5-CH_2); 4.06 (m, 2H, CH_2OH); 6.95–7.38 (m, 5H, arom); Anal. ($C_{12}H_{14}N_2O_2S$) C, H, N.

6.3. General procedures for the synthesis of 5-arylidene-3-hydroxyalkyl-2-phenylimino-4-thiazolidinones 7–8a, c–e

The appropriate benzaldehyde (30 mmol) was added to a solution of **5** or **6** compounds (30 mmol) and piperidine (50 mmol) in ethanol (35 mL) and the mixture was heated at reflux for 24 h. After this time, the solution was concentrated in vacuo and the residue was recrystallized from methanol.

6.3.1. 3-(2-Hydroxyethyl)-5-(4-methylthiophenyl)methylidene-2-phenylimino-4-thiazolidinone (7a). Yield 65%; mp 161–163 °C; ^{1}H NMR ($CDCl_3$): δ 2.50 (s, 3H, SCH_3); 4.01 (t, 2H, NCH_2, J = 4.2 Hz); 4.29 (m, 2H, CH_2OH); 7.04–7.44 (m, 9H, arom); 7.74 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 15.0 (SCH_3); 46.0 (NCH_2); 61.5 (CH_2OH); 120.0 (5-C); 121.4, 125.0, 125.7, 129.4, 130.3 (CH arom); 131.2 (CH methylidene); 129.7, 142.2, 147.4, 152.7 (Cq); 176.2 (CO); Anal. ($C_{19}H_{18}N_2O_2S_2$) C, H, N.

6.3.2. 3-(2-Hydroxyethyl)-5-(3-methoxyphenyl)methylidene-2-phenylimino-4-thiazolidinone (7c). yield 65%; mp 91–93 °C; ^{1}H NMR ($CDCl_3$): δ 3.82 (s, 3H, OCH_3); 4.01 (t, 2H, NCH_2, J = 4.8 Hz); 4.3 (t, 2H, CH_2OH, J = 4.8 Hz); 6.92–7.44 (m, 9H, arom); 7.76 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 46.1 (NCH_2); 55.2 (OCH_3); 61.6 (CH_2OH); 115.3, 115.5, 121.3, 122.1, 125.2, 129.4, 131.7 (CH arom); 121.6 (5-C); 130.4 (CH methylidene); 134.8, 147.3, 152,5, 159,9 (Cq); 167.1 (CO); Anal. ($C_{19}H_{18}N_2O_3S$) C, H, N.

6.3.3. 5-(4-Chlorophenyl)methylidene-3-(2-hydroxyethyl)-2-phenylimino-4-thiazolidinone (7d). Yield 62%; mp 140–142 °C; ^{1}H NMR ($CDCl_3$): δ 4.00 (t, 2H, NCH_2, J = 4.2 Hz); 4.28 (m, 2H, CH_2OH); 7.02–7.85 (m, 9H, arom); 7.73 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 46.1 (NCH_2); 61.3 (CH_2OH); 121.1, 125.6, 129.1, 129.5, 131.3 (CH arom); 121.9 (5-C); 130.7 (CH methylidene); 132.1, 136.0, 147.3, 152,2 (Cq); 167.0 (CO); Anal. ($C_{18}H_{15}ClN_2OS$) C, H, N.

6.3.4. 3-(2-Hdroxyethyl)-5-(4-methoxyphenyl)methylidene-2-phenylimino-4-thiazolidinone (7e). Yield 64%; mp 135–137 °C; ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H, OCH_3); 4.00 (t, 2H, NCH_2, J = 4.8 Hz); 4.30 (m, 2H, CH_2OH); 6.90–7.43 (m, 9H, arom); 7.77 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 46.2 (NCH_2); 55.5 (OCH_3); 61.6 (CH_2OH); 114.7, 121.7, 125.6, 129.3, 132.2 (CH arom); 117.8 (5-C); 131.9 (CH methylidene); 126.2, 146.6, 154.0, 161.2 (Cq); 167.1 (CO); Anal. ($C_{19}H_{18}N_2O_2S$) C, H, N.

6.3.5. 3-(3-Hydroxypropyl)-5-(4-methylthiophenyl)methylidene-2-phenylimino-4-thiazolidino-ne (8a). Yield 62%; mp 120–122 °C; ^{1}H NMR ($CDCl_3$): δ 2.00 (m, 2H, CH_2); 2.51 (s, 3H, SCH_3); 3.69 (t, 2H, NCH_2, J = 5.4 Hz); 4.20 (m, 2H, CH_2OH); 7.04–7.46 (m, 9H, arom); 7.74 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 14.9 (SCH_3); 30.6 (CH_2); 39.5 (NCH_2); 58.3 (CH_2OH); 119.7 (5-C); 121.3, 125.3, 126.0, 129.5, 130.3 (CH arom); 131.1 (CH methylidene); 129.7, 142.3, 147.4, 152.6 (Cq); 167.2 (CO); Anal. ($C_{20}H_{20}N_2O_2S_2$) C, H, N.

6.3.6. 3-(3-Hydroxypropyl)-5-(3-methoxyphenyl)methylidene-2-phenylimino-4-thiazolidinone (8c). Yield 65%; mp 90–92 °C; ^{1}H NMR ($CDCl_3$): δ 2.00 (m, 2H, CH_2); 3.68 (t, 2H, NCH_2, J = 5.7 Hz); 3.81 (s, 3H, OCH_3); 4.21 (m, 2H, CH_2OH); 6.92–7.44 (m, 9H, arom); 7.76 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 30.6 (CH_2); 39.6 (NCH_2); 55.2 (OCH_3); 58.6 (CH_2OH); 115.3, 115.5, 121.1, 122.1, 125.2, 129.3, 131.5 (CH arom); 121.6 (5-C); 130.1 (CH methylidene); 134.8, 147.4, 152.4, 159,9 (Cq); 167.1 (CO); Anal. ($C_{20}H_{20}N_2O_3S$) C, H, N.

6.3.7. 5-(4-Chlorophenyl)methylidene-3-(3-hydroxypropyl)-2-phenylimino-4-thiazolidinone (8d). Yield 61%; mp 99–101 °C; ^{1}H NMR ($CDCl_3$): δ 2.00 (m, 2H, CH_2); 3.68 (t, 2H, NCH_2, J = 5.7 Hz); 4.21 (m, 2H, CH_2OH); 7.02–7.45 (m, 9H, arom); 7.72 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 31.0 (CH_2); 40.0 (NCH_2); 58.7 (CH_2OH); 121.5, 125.6, 129.7, 130.3, (CH arom); 122.2 (5-C); 131.4 (CH methylidene); 132.3, 136.3, 147.7, 152.2 (Cq); 167.2 (CO); Anal. ($C_{19}H_{17}ClN_2OS$) C, H, N.

6.3.8. 3-(3-Hydroxypropyl)-5-(4-methoxyphenyl)methylidene-2-phenylimino-4-thiazolidinone (8e). Yield 65%; mp 129–131 °C; ^{1}H NMR ($CDCl_3$): δ 1.98 (m, 2H, CH_2); 3.66 (t, 2H, NCH_2, J = 5.7 Hz); 3.83 (s, 3H, OCH_3);

4.20 (m, 2H, CH_2OH); 6.92–7.42 (m, 9H, arom); 7.74 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 30.9 (CH_2); 39.4 (NCH_2); 55.3 (OCH_3); 58.4 (CH_2OH); 114.7, 121.3, 125.9, 129.4, 131.9 (CH arom); 118.2 (5-C); 131.5 (CH methylidene); 126.1, 147.6, 152.8, 161.0 (Cq); 167.4 (CO); Anal. ($C_{20}H_{20}N_2O_2S$) C, H, N.

6.4. Synthesis of 3-hydroxyalkyl-5-(4-methylsulfonylphenyl)methylidene-2-phenylimino-4-thiazolidinone 7b, 8b

A suspension of compound **7a** or **8a** (0.2 mol) in 30 mL acetic acid (50%) was treated dropwise with H_2O_2 35% (0.3 mol, 30 g) and stirred for 48 h at room temperature. The mixture was then filtered and the residue was treated dropwise with H_2O_2 35% (0.3 mol, 30 g) and stirred for 48 h at room temperature. The residue was filtered and recrystallized from methanol.

6.4.1. 3-(2-Hydroxyethyl)-5-(4-methylsulfonylphenyl)methylidene-2-phenylimino-4-thiazolidinone (7b). Yield 93%; mp 208–210 °C; ^1H NMR ($CDCl_3$): δ 3.01 (s, 3H, SO_2CH_3); 3.9 (t, 2H, NCH_2, $J = 5.1$ Hz); 4.00 (m, 2H, CH_2OH); 7.68–8.06 (m, 9H, arom); 7.93 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 44.1 (SO_2CH_3); 46.3 (NCH_2); 61.7 (CH_2OH); 125.7 (5-C); 121.2, 125.0, 125.6, 128.0, 129.5 (CH arom); 130.3 (CH methylidene); 138.7, 141.0, 147.0, 151.8, (Cq); 167.2 (CO); Anal. ($C_{19}H_{18}N_2O_4S_2$) C, H, N.

6.4.2. 3-(3-Hydroxypropyl)-5-(4-methylsulfonylphenyl)methylidene-2-phenylimino-4-thiazolidinone (8b). Yield 92%; mp 101–103 °C; ^1H NMR ($CDCl_3$): δ 2.05 (m, 2H, CH_2); 3.06 (s, 3H, SO_2CH_3); 3.68 (t, 2H, NCH_2, $J = 5.7$ Hz); 4.21 (m, 2H, CH_2OH); 7.01–7.99 (m, 9H, arom); 7.79 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 30.5 (CH_2); 39.9 (NCH_2); 44.4 (SO_2CH_3); 58,4 (CH_2OH); 125.5 (5-C); 121.2, 124.2, 125.5, 127.9, 129.5 (CH arom); 130.3 (CH methylidene); 138.7, 140.9, 147.1, 151.1 (Cq); 166.4 (CO); Anal. ($C_{20}H_{20}N_2O_4S_2$) C, H, N.

7. Cell isolation and human articular chondrocyte culture

Human articular cartilage was obtained at replacement surgery from patients with femoral neck fractures. The isolation procedure was conducted under aseptic conditions. The cartilage was cut into small fragments and carefully washed using Dulbecco's Modified Eagle's Medium (DMEM) culture medium containing $NaHCO_3$, 25 mM Hepes, 1 mM sodium piruvate, 50 mg/mL gentamycin, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 mg/mL amphotericin B. Chondrocytes were isolated through 3 sequential passages of enzymatic digestion of the extracellular matrix. Incubation with 0.1% hyaluronidase type III (1 mg/mL for 100 mg of cartilage), for 30 min at 37 °C; incubation with 0.5% pronase type XIV (5 mg/mL for 100 mg of cartilage), for 60 min at 37 °C; finally incubation with 0.2% collagenase type IA (2 mg/mL for 100 mg of cartilage), for 45 min at 37 °C. The cellular suspension obtained was filtered (filters from 100 and 70 mm) to eliminate the residues of the digestion and the cellular aggregates and to obtain a monocellular suspension of chondrocytes. This was washed 3 times with DMEM supplemented with 10% foetal calf serum (FCS), and was subjected to vital colouration method staining with eosin in order to determine the number and the vitality of recovered cells. After 24 h the medium was removed and cells were treated as follows: (a) Control, (b) IL-1β (10 ng/mL), (c) Indomethacin (10^{-5} M) + IL (10 ng/mL), d–h) **7a**–**7e** (10, 50 and 100 μg/mL) + IL (10 ng/mL), i–m) **8a**–**8e** (10, 50 and 100 μg/mL) + IL (10 ng/mL), n) **2** (10, 50 and 100 μg/mL) + IL (10 ng/mL).

After 120 h the supernatants of cartilage culture were collected for different assays.

7.1. Cell viability assay

The cytotoxic effect of the experimental substances was evaluated by a cell viability test based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells.[5,6,17]

7.2. Determination of nitrite levels

Nitrite was determined by adding 100 μl of Griess reagent (1% sulfanylamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 5% of hydrochloric acid) to 100 μl of samples.[18] The optical density at $\lambda = 570$ nm was measured using a microtitre plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite prepared in medium.

7.3. Determination of glycosaminoglycans (GAGs)

The level of GAGs was measured by spectrophotometry with a solution of 1.9-dimethylmethylene blue at $\lambda = 535$ nm.[19] The amount of glycosaminoglycans was calculated from a standard curve obtained for shark chondroitin sulfate.

7.4. Determination of prostaglandins (PGE_2)

PGE_2 was determined in the culture supernatant by the enzyme immunoassay (EIA) system using a commercially available immunoassay kit (Amersham Pharmacia, UK) according to the manufacturer's instructions. The detection limit is 1 pg/mL. The values were expressed as pg/mL PGE_2 released.

7.5. Determination of MMP-3

MMP-3 was determined in the culture supernatant by the ELISA system using a commercially available immunoassay kit (Amersham-Pharmacia, UK) according to the manufacturer's instructions. The detection limit is 1 ng/mL. The values were expressed as ng/mL MMP-3 released.

7.6. Statistical analysis

All the present results 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. A value of $P < 0.05$ was considered to be statistically significant.

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