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Heteroarylimino-4-thiazolidinones as inhibitors of cartilage degradation

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ABSTRACT

2-Benzo[*d*]thiazolyl- and 2-benzo[*d*]isothiazolyl-imino-5-benzylidene-4-thiazolidinone derivatives were investigated as potential metalloproteinases (MMPs) inhibitors and evaluated for their antidegenerative activity on human chondrocyte cultures stimulated by IL-1β, using an experimental model that reproduces the mechanisms involved in osteoarthritic (OA) diseases. Cell viability, the amount of glycosaminoglycans (GAGs) and the production of nitric oxide (NO) were measured. The most potent compound, 5-(4-methoxy-benzylidene)-2-(benzo[*d*]isothiazol-3-ylimino)-thiazolidin-4-one (**4b**), a MMP-13 inhibitor at nanomolar concentration (IC_{50} = 0.036 μM), could be considered as a lead compound for the development of novel clinical agents, inhibitors of cartilage degradation, for the treatment of OA.

1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that have been implicated in a wide variety of biological processes and pathological conditions such as cancer, heart failure, atherosclerosis and arthritis [1–4]. Increased levels of these enzymes, that are responsible for degradation of aggrecan and collagen, have been observed in the cartilage of patients with osteoarthritis (OA) and correlate with the severity of the disease.

OA is a multifactorial disease involving a progressive destruction of joint tissue and cartilage through a complex series of mechanisms. Cartilage consists of a relatively small number of chondrocytes and many extracellular matrix (ECM) components, comprising mainly collagen fibrils and aggrecan (a large aggregating proteoglycan). Chondrocytes synthesize and catabolise ECM macromolecules, while the matrix in turn maintains the homeostasis of the cellular environment and cartilage structure. In OA diseases, the degradation of the ECM exceeds its synthesis, resulting in a net decrease in the amount of cartilage matrix. The deregulated synthesis of matrix components involves key molecules in the erosion of cartilage, such as proteoglycan, aggrecan and type II collagen, whose loss and destruction have recently been recognized as significant factors in OA [5]. The primary cause of this process is an increase in the activities of the matrix metalloproteinases (MMPs), a family of structurally related zinc-dependent endopeptidases collectively capable of degrading all ECM components, in pathological situations as OA.

Currently, approximately 27 MMPs are known, grouped into subfamilies of collagenases, gelatinases, stromelysins, membrane-type MMPs, and novel MMPs, on the basis of their structure and substrate specificity [6–8]. The main MMPs involved in the pathogenesis of OA are collagenases, stromelysins and gelatinases with the ability to degrade triple helix collagen, proteoglycans and denatured collagen, respectively [9]. Stromelysins are recognized as collagenase-related connective tissue-degrading metalloproteinases capable of degrading ECM multiple components, or stroma. Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) isoforms are similar in their amino acid sequence and substrate specificity. MMP-3, stromelysin-1, is particularly destructive towards matrix components including the protein structure of proteoglycans [10,11]. MMP-13, collagenase, consistently found in the deeper layers appears to be the major mediator of type II collagen destruction [12–14]. Members of the collagenase subgroup of MMPs, *i.e.* collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13), are the principal neutral proteinases capable of degrading native fibrillar collagens in the extracellular space. They all cleave types I, II, and III collagens; MMP-13 cleaves fibrillar collagens with preference to type II collagen over types I and III collagens.

In addition, the functional alteration of cartilage also results from the intervention of different cytokines, especially IL-1β. It induces high levels of pro-inflammatory mediators, such as prostaglandins E_2 (PGE_2) and nitric oxide (NO), and inhibits collagen and proteoglycan synthesis. IL-1β is a pivotal driving force in

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inducing and sustaining cartilage damage that usually leads to the loss of sulfated glycosaminoglycans (GAGs) [15]. GAG release is a consequence of increased matrix protease activity, leading to the cleavage of collagen and proteoglycans. High levels of PGE_2 also mediate cartilage resorption by decreasing chondrocyte proliferation, enhancing MMPs activity and inhibiting aggrecan synthesis in chondrocytes [16]. During inflammatory processes, that represent one of the key pathogenetic aspects of degenerative joint disease, PGE_2 production is intensified and this contributes to synovial inflammation by increasing local blood flow and by potentiating the effects of mediators such as bradykinin, responsible for relevant vasopermeability. It has been shown that PGE_2 is able to inhibit chondrocyte growth and upregulate IL-1β [17]. The above findings demonstrate the importance of these mediators in human OA.

The pharmacological formulary currently possesses few weapons against joint destruction [18,19] indicating the need for development of molecules capable of such inhibitory activity. As a part of an intensive research program focused on the design and synthesis of new chondroprotective/anti-inflammatory drugs [20,21], our interest in this field prompted us to study 4-thiazolidinone derivatives 1a–6a and 1b–**6b** (Table 1) belonging to a chemical class of molecules, the 4-thiazolidinones, that have been demonstrated to possess anti-inflammatory properties and a protective action on inflammatory degenerative diseases [22–24], such as OA.

We here report the MMP-3 and MMP-13 inhibition capability (IC_{50}) and the antidegenerative/chondroprotective effect, determined by evaluating cell viability, NO and GAGs levels of 2-(benzo[*d*]-thiazol-2-ylimino)-thiazolidin-4-one (**1a**), 2-(benzo[*d*]thiazol-2-ylimino)-5-benzylidenethiazolidin-4-ones (**2a**–**6a**) and of their isomers 2-(benzo[*d*]isothiazol-3-ylimino)-thiazolidin-4-one (**1b**) and 2-(benzo[*d*]isothiazol-3-ylimino)-5-benzylidenethiazolidin-4-ones (**2b**–**6b**). In order to find the pharmacophore and explore the structure–activity relationships of these compounds we considered simple substitutions of the benzylidene moiety based on previous SAR studies.

2. Materials and methods

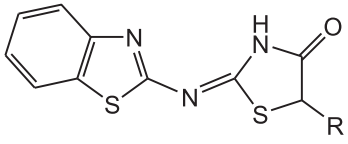
2.1. Chemistry

The general synthetic approach to heteroarylimino-4-thiazolidinones is shown in Fig. 1. The compounds described here (Table 1) were synthesized by the multi-step reaction protocol reported earlier by us [25].

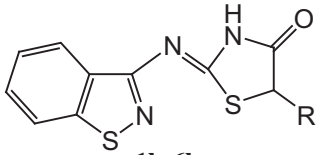
2.2. MMP-3 and MMP-13 fluorimetric assay

The compounds were evaluated for their ability to inhibit the hydrolysis of fluorescence-quenched peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Biomol, Inc.). The assays were performed in 50 mM HEPES buffer for MMP-13, MES 50 mM only for

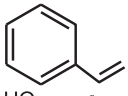
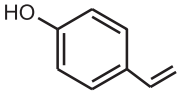
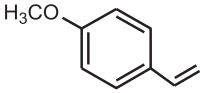
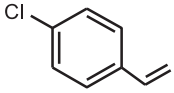
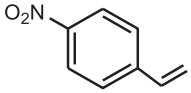
Table 1
MMP-3 and MMP-13 inhibitory activity of compounds synthesized.



1a-6a



1b-6b

R-	Comp	MMP-3, IC_{50} (μM)	MMP-13, IC_{50} (μM)	Comp	MMP-3, IC_{50} (μM)	MMP-13, IC_{50} (μM)
H	**1a**	36.68	n.a.[a]	**1b**	n.a.[a]	n.a.[a]
	**2a**	44	45	**2b**	n.a.[a]	32
	**3a**	n.a.[a]	n.a.[a]	**3b**	10.35	0.158
	**4a**	16.7	98	**4b**	n.a.[a]	0.036
	**5a**	65	20	**5b**	65	25
	**6a**	n.a.[a]	60	**6b**	n.a.[a]	55.88

Representative results of at least three independent experiments are reported.
[a] Not active: IC_{50} (μM) > 100.

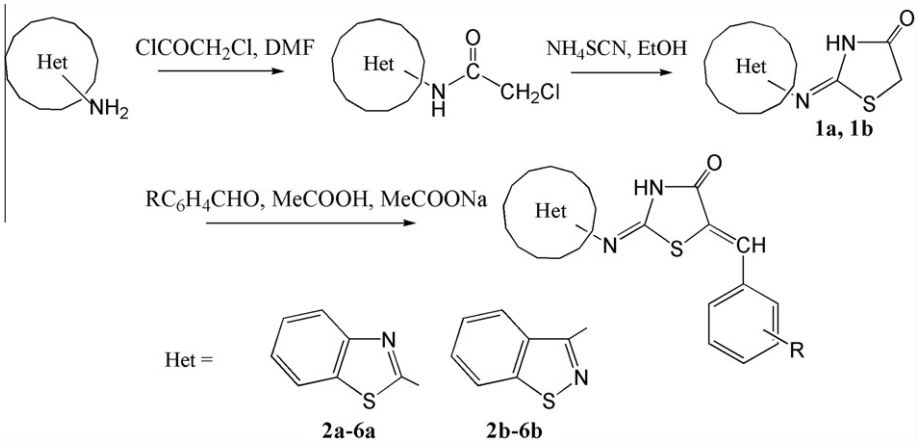


Fig. 1. General synthetic scheme.

MMP-3, containing 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.05% Brij-35, at pH 7, using 10 nM of proteolytic enzyme (catalytic domains of MMP-3 and MMP-13) (Biomol, Inc.) and 350 nM of peptide. The enzyme was incubated at 25 °C with increasing concentration of the inhibitor and the fluorescence (excitation$_{max}$ 328 nm; emission$_{max}$ 393 nm) was measured for 3 min after the addition of the substrate using a Varian Eclipse fluorimeter. Fitting of rates as a function of inhibitor concentration provided IC_{50} values. The inhibitor *N*-isobutyl-*N*-[4-methoxyphenylsulfonyl]glycyl hydroxamic acid (Biomol, Inc.) was used as control [26].

2.3. Cell isolation and human articular chondrocyte culture

Normal human articular cartilage was obtained at replacement surgery from some patients with femoral neck accidental fractures and that informed consent was obtained. The isolation procedure was conducted under antiseptic conditions. The cartilage was cut into small fragments and carefully washed using Dulbecco's Modified Eagles Medium (DMEM) culture medium containing $NaHCO_3$, 25 mM Hepes, 1 mM sodium pyruvate, 50 mg/mL gentamycin, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 mg/mL amphotericin B. Chondrocytes were isolated through three 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 obtained cellular suspension was filtered (filters from 100 and 70 mm) to eliminate the residues of the digestion and cellular aggregates. Freshly isolated chondrocytes were seeded into monolayer culture at a cell density of 2×10^5 cells and cultured in 1 mL of DMEM supplemented with 10% fetal bovine serum (FCS) at 37 °C in 5% CO_2/95% air. Confluent chondrocytes of primary culture were used for all the experiments: the tested compounds were assayed dissolved in DMSO, appropriately diluted in Dulbecco's Modified Eagle's Medium (DMEM) and dispensed to the wells to give to different concentrations, from 10 μg/mL to 0.01 μg/mL. IL-1β was used at 10 ng/mL alone or combined with experimental compounds. The assays were performed after 120 h of treatment.

2.4. 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 [27].

2.5. Determination of nitrite

Nitrite was determined by adding 100 μL of Griess reagent (1% sulphanylamide and 0.1% naphthylethylenediamine dihydrochloride in 5% of hydrochloric acid) to 100 μL of samples [28]. The optical density at λ = 570 nm was measured using a microtiter plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite standard curve (0–120 μM).

2.6. Determination of glycosaminoglycans

The level of GAGs, an index of cartilage damage, was measured by spectrophotometry with a solution of 1,9-dimethylmethylene blue at λ = 535 nm using the method of Farndale et al. [29]. The amount of glycosaminoglycans was calculated from a standard curve (100–500 μg/mL) obtained for shark chondroitin sulfate C, derived from shark cartilage.

2.7. 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. $P < 0.05$ was considered to be statistically significant. The statistical analysis was performed by using one-way ANOVA followed by Dunnett's post hoc test for multiple comparison with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA).

3. Results and discussion

As shown in Table 1, many of the tested compounds displayed inhibitory activity (IC_{50}) against MMP-3 and MMP-13. The MMP assays were performed evaluating the ability of compounds **1a**–**6a** and **1b**–**6b** to prevent the hydrolysis of the fluorescence-quenched peptide substrate Mca-Pro-Gly-Leu-Dpa-Ala-Arg-NH_2. Marked differences in potency and selectivity were observed for the analogs with different core structures (benzo[*d*]thiazol-3-ylimino or benzo-[*d*]isothiazol-3-ylimino). The highest activity and selectivity for MMP-13 was exhibited by the group of 2-(benzo[*d*]isothiazol-3-ylimino)-5-benzylidenethiazolidin-4-ones (**2b**–**6b**). The 5-(4-hydroxybenzylidene)-2-(benzo[*d*]isothiazol-2-ylimino)thiazolidin-4-one

(**3b**) and 5-(4-methoxybenzylidene)-2-(benzo[*d*]isothiazol-2-ylimino)thiazolidin-4-one (**4b**) derivatives resulted the most potent MMP-13 inhibitors with IC_{50} value of 0.158 and 0.036 μM, respectively. The structure–activity relationship analysis of the data reported in Table 1 shows also that the parent compounds were inactive (**1a** against MMP-13 and **1b** against both the enzymes: $IC_{50} > 100$ μM), or moderately active (**1a** for MMP-3, $IC_{50} = 39.68$ μM), suggesting that the unsubstituted and substituted 5-arylidene moiety plays an important role in enhancing the inhibitory activity of MMPs. Among the 5-arylidene derivatives, the inhibitory effect appears to be dependent on the substitution at the benzene ring. The introduction of the hydrophilic, electron-donating hydroxy group at the para position of the benzylidene moiety (**3b**) increased, in the benzisothiazolylimino class, the inhibitory potency against MMP-13 and MMP-3 with IC_{50} of 10.35 and 0.158 μM, respectively. In contrast, the benzothiazolylimino isomer (**3a**) did not show inhibition, for both MMP-3 and MMP-13, at or below 100 μM. The introduction of the moderately hydrophilic, electron-donating methoxy group at the benzylidene para position in the benzisothiazolylimino analog (**4b**) resulted in $IC_{50} > 100$ μM against MMP-3 and in the highest increase in activity on MMP-13 ($IC_{50} = 0.036$ μM); notably, its benzothiazolylimino isomer (**4a**) displayed a sixfold selectivity for MMP-3 over MMP-13 (IC_{50} 16.7 versus 98 μM). The introduction of the lipophilic/electron-withdrawing chloro group in the isomeric compounds **5a** and **5b**, afforded increase of potency, against MMP-13 with respect to the unsubstituted analogs, as well as against MMP-3 for **5b**, while a slight reduction of activity was observed in the activity of **5a** on MMP-3. A similar effect was observed between the isomeric compounds **6a** and **6b** bearing the nitro electron-withdrawing group at the benzylidene para position; with respect to their chloro analogs they showed a significant enzyme selectivity for MMP-13 (IC_{50} 60 and 55.88 μM) over MMP-3 ($IC_{50} > 100$ μM). This suggests that the electronic and hydrophilic/lipophilic effects of the substituents on the benzene ring plays an important role in the modulation of MMPs inhibitory activity and hydrophilic, electron-donating groups seem to favor the potency and the selectivity as shown by compounds **3b** and **4b** against MMP-13.

The compounds were in vitro assayed on chondrocytes at different concentrations from 10 μg/mL to 0.01 μg/mL. The results are reported at 10 μg/mL, representing the best data for cellular assays, probably due to the different behavior of the compounds on biological systems. The tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorogenic assay was chosen as the method for quantifying cell viability. All tested compounds did not reduce the ability of chondrocytes to metabolise tetrazolium salts, demonstrating that they did not interfere with cell viability (data not shown).

Fig. 2 shows NO production levels by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **1a–6a**, and their isomers **1b–6b** at a concentration of 10 μg/mL. The tested compounds strongly inhibited NO production induced by inflammatory IL-1β. The 4-chlorosubstituted derivatives (**5a**, **5b**) and the 4-nitrosubstituted compounds (**6a**, **6b**) of both benzothiazolylimino and benzisothiazolylimino series, when combined with IL-1β, showed a significant reduction in NO release, compared to the sample treated only with IL-1β (21.68 μM). In particular, compound **5a** proved to be the most efficacious derivative (4.87 μM), as well as its isomer **5b** (9.55 μM). Furthermore, compound **6b** exhibited a level of NO production of 5.18 μM, while its isomer (**6a**) showed a level of NO production of 9.15 μM. This indicates that insertion of lipophilic, electron-withdrawing groups at the benzylidene para position is the most favorable substitution to decrease the NO production levels. It is worth noting that the

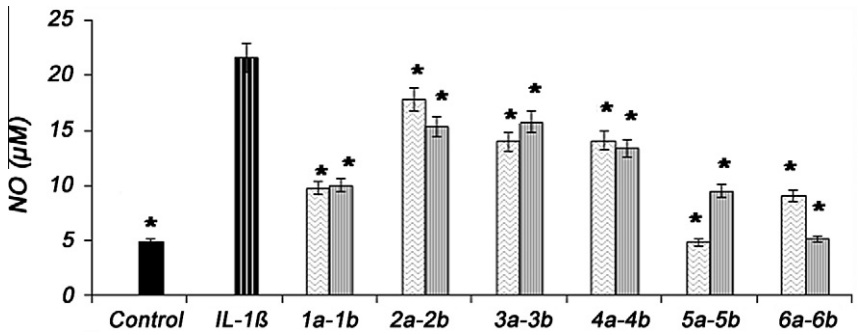


Fig. 2. Effect of heteroarylimino-4-thiazolidinones on NO release. NO production (μM) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **1a**–**6a** and their isomers **1b**–**6b** at 10 μg/mL. $^*P < 0.005$.

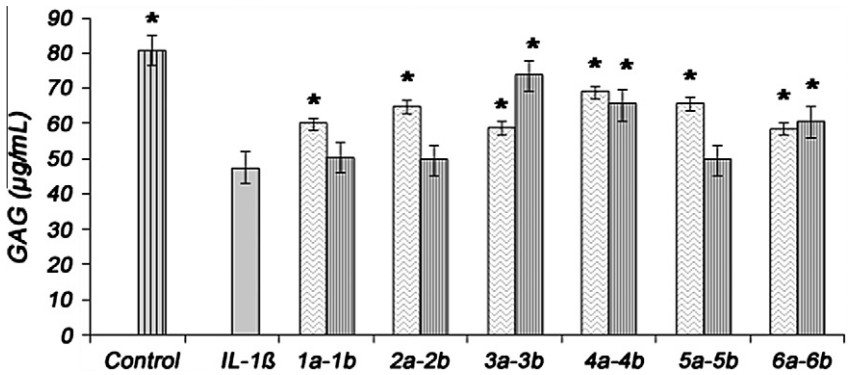


Fig. 3. Effect of heteroarylimino-4-thiazolidinones on GAGs release. GAGs release (μg/mL) (means ± SEM) in the culture medium by IL-1β stimulated articular chondrocytes 120 h after the addition of compounds **1a**–**6a** and their isomers **1b**–**6b** at 10 μg/mL. $^*P < 0.005$.

parent compound **1a** and its isomer **1b** also exhibited good anti-degenerative/anti-inflammatory activity being able to reduce the NO levels induced by the pro-inflammatory IL-1β. It is noteworthy that all the compounds inhibited the NO production induced by IL-1β.

The level of GAGs was measured by the spectrophotometric method of Farndale et al. that represents an index of cartilage damage in chondrocyte cultures [30,31]. As can be seen in Fig. 3, all compounds **1a**–**6a**, and their isomers **1b**–**6b**, combined with IL-1β, showed inhibition of IL-1β activity. At 10 μg/mL all the tested compounds had values close to those of the untreated controls, by preventing the depletion of proteoglycan. Compound **3b** increased the release of GAGs of 43% and its isomer **3a** increased the release of GAGs by 34.81% compared to the IL-1β. Compound **4b**, with a methoxy group at the benzylidene para position, increased the release of GAGs by 38%, compared to IL-1β, while its isomer **4a** increased the release of the GAGs by 40.7%.

Surprisingly, even though the molar concentration of the compounds **3b** and **4b** in cellular based assays was far above (27–28 μM) their sub-micromolar IC_{50} values against MMP-13, they showed a moderate effect on GAGs and NO release, comparable to the effect of heteroarylimino-4-thiazolidinones less active as MMPs inhibitors, that seems to indicate that the reason of the above inflammation mediators release is independent from enzyme inhibition. On the other hand it is evident that lipophilic substituents like chloro and nitro groups, improving cell penetration, favor the chondroprotective effect in cell based assays and possible detrimental effect of the hydrophilic OH and OCH_3 groups of **3b** and **4b** on cell penetration might explain their relatively modest effect on GAG and NO release.

Notwithstanding this, it is of note that the paramethoxy and parahydroxy 5-benzylidene substitutions on the benzisothiazolylimino-4-thiazolidinone are favorable for the inhibition of all the inflammation/chondrodestruction mediators tested, with a ≈1000 fold increase in MMP-13 inhibitory activity for compound **4b** demonstrating that in particular 5-(4-methoxy-benzylidene)-2-(benzo[*d*]isothiazol-3-ylimino)-thiazolidin-4-one (**4b**) could counteract the harmful effects induced by IL-1β.

4. Conclusions

Two isomeric classes of heteroarylimino-4-thiazolidinones have been explored and were found to be inhibitors of MMPs and degenerative/inflammation mediators, such as NO and GAGs. The novelty of the 4-thiazolidinone scaffold for the MMPs activity represents the main finding of the present study and the most potent compound, 5-(4-methoxy-benzylidene)-2-(benzo[*d*]isothiazol-3-ylimino)-thiazolidin-4-one (**4b**), a MMP-13 inhibitor at nanomolar concentration (IC_{50} 0.036 μM) could be considered a lead compound for the development of novel agents, inhibitors of cartilage degradation, for the treatment of OA. Further exploration of the SAR of these novel MMP inhibitors is under evaluation and will be reported in due course.

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