Benzisothiazolyliminothiazolidin-4-ones with chondroprotective properties: searching for potent and selective inhibitors of MMP-13.

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Benzisothiazolyliminothiazolidin-4-ones with Chondroprotective Properties: Searching for Potent and Selective Inhibitors of MMP-13

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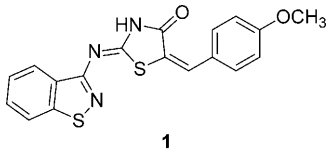
Matrix metalloproteinase-13 (MMP-13) plays a key role in the degradation of type II collagen in cartilage and bone in osteoarthritis.[1] Nonselective inhibition of different MMPs by the early inhibitors appears to be the reason for their toxicity and limited efficacy.[2] Recent findings suggest that selective inhibition of MMP-13 could avoid toxicity and that non-zinc-chelating MMP inhibitors appear to be the most promising agents toward achieving selectivity, since the allosteric binding sites are not shared by different MMPs. Biochemical, histological and clinical models support this findings. [1b,3] Therefore, effective MMP-13 inhibition would be a novel, disease-modifying therapy for the treatment of osteoarthritis.

Our recent efforts in the search for new agents with antidegenerative activity, as evaluated in human chondrocyte cultures stimulated by IL-1β, have resulted in the discovery of a series of heteroarylimino-4-thiazolidinones that significantly inhibit MMPs and other inflammatory mediators.[4] Among these, a potent and selective MMP-13 inhibitor has been identified (MMP-13, $IC_{50} = 0.036$ μM; MMP-3, $IC_{50} > 100$ μM) based upon a 2-(benzo[*d*]isothiazol-3-ylimino)-5-(4-methoxybenzylidene)thiazolidin-4-one scaffold (**1**; Figure 1). The presence of the electron-donating, moderately hydrophilic and bulky methoxy group at the benzylidene *para* position in compound **1** afforded the highest improvement in MMP-13 inhibition among all the heteroarylimino analogues of the series. This prompted us to explore the structure–activity relationship within the three positional methoxybenzylidene isomers **1**–**3** and evaluate the binding mode between compounds **1**–**3** and MMP-13 by molecular modeling.



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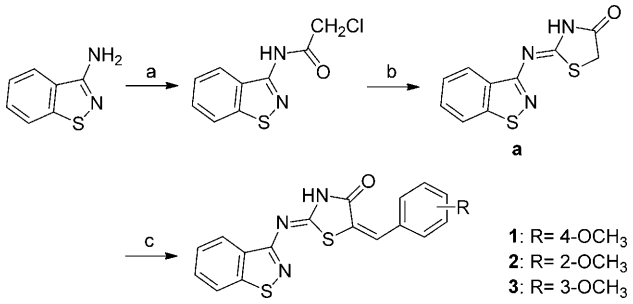
IC_{50} = 36 nM (MMP-13)

Figure 1. 2-(Benzo[*d*]isothiazol-3-ylimino)-5-(4-methoxybenzylidene)thiazolidin-4-one (**1**) structure and MMP-13 inhibitory activity.[4]

Herein, we describe the synthesis, the in vitro evaluation and the docking studies of compounds **1**–**3**. The synthesis of the studied compounds was accomplished as illustrated in Scheme 1. Target compounds **1**–**3** were prepared from 2-(benzo[*d*]isothiazol-3-ylimino)thiazolidin-4-one (**a**; Scheme 1), recently synthesized in our laboratory, by reaction with the appropriately substituted aryl aldehyde according to previously reported protocols.[5]



Scheme 1. *Reagents and conditions*: a) $ClCOCH_2Cl$, DMF, RT, 2 h, 72%; b) NH_4SCN, EtOH, reflux, 3 h, 76%; c) RC_6H_4CHO, CH_3COOH, CH_3COONa, reflux, 4–6 h, 76–89%.

MMP-13 inhibition (IC_{50}) was investigated by evaluating the ability of compounds **1**–**3** to prevent the hydrolysis of the appropriate fluorescence-quenched peptide substrate.[6] To get a deeper insight into the mechanism of chondroprotective action of compounds **1**–**3**, their effect in cultures of human chondrocytes, stimulated by IL-1β, was evaluated by determining cell viability, nitric oxide (NO) and glycosaminoglycan (GAG) levels.[1c] In addition, the antioxidant properties of the compounds were tested by means of an oxygen radical absorbance capacity (ORAC) assay and by determining the free radical scavenging ability of the molecules through measuring the extent of their interaction with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH).[7]

As shown in Table 1, modification of the methoxy substituent position afforded a significant decrease in the MMP-13 inhibitory activity of compounds **2** and **3** ($IC_{50} > 100$ μM) compared with **1** ($IC_{50} = 0.036$ μM), while retaining the cartilage antidegenerative profile. Compound **1** has high potency against MMP-13 and selectivity over MMP-3, in contrast to compounds **2** and **3**, suggesting that the methoxy-substituted 5-arylidene moiety plays an important role depending on the substituent position on the benzene ring. The chondroprotective effect of

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Table 1. Effects of 2-(benzo[*d*]isothiazol-3-ylimino)-5-(methoxybenzylidene)thiazolidin-4-ones **1–3**.

Compd	R	GAGs[a] (% increase)	NO[a] (% decrease)	IC_{50} [μM] MMP-3	MMP-13	DPPH[b] [%]	ORAC[c]
1	4-OCH_3	37±4	37±2	>100	0.036	n.a.[d]	n.a.[d]
2	2-OCH_3	17±2	42±4	89.3	>100	n.a.[d]	n.a.[d]
3	3-OCH_3	11±0.8	59±2	>100	>100	91	49

[a] Expressed as % increase or decrease compared to IL-1β. [b] Expressed as % inhibition. [c] Expressed as trolox equivalents per gram. [d] Not active.

isomers **1**–**3** was evaluated at 10 μM in culture of human chondrocytes stimulated by IL-1β. Table 1 reports the NO reduction of the release obtained 120 h after the addition of compounds **1**–**3**. 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 alone. Compounds **1**–**3** were all able to decrease the NO production; in particular, compound **3** showed a reduction in release of NO by 59%. IL-1β is involved in the degenerative process and induces a reduction in GAGs that, with collagen and proteoglycans, form macromolecular aggregates making up the extracellular matrix (ECM). All test compounds (**1**–**3**) enhanced, to different extents, the release of IL-1β-induced GAGs; in particular, compound **1** increased the release of GAGs by 37% compared to IL-1β-treated chondrocytes, demonstrating its ability to restore normal levels of GAGs available for the synthesis of proteoglycans.

The relationship between free radicals, such as NO that are also responsible for the develpment of chondrodegenerative diseases, and the radical scavenging activity of compound **1**–**3** was also investigated. This was achieved by measuring the antioxidant activity in vitro by means of a DPPH test, and then confirmed using ORAC assay, using quercetin and trolox as standards. These methods are regarded as good in vitro models for the determination of the effectiveness and suitability of potential antioxidant/chondroprotective compounds. In Table 1, as demonstrated by the scavenging activity shown in the DPPH test and confirmed in the ORAC assay, only compound **3** showed potent free radical scavenging activity. This compound is the only isomer that exhibits significant activity as a NO inhibitor. The inhibition of multiple radicals can decrease the incidence rate of chronic diseases such as osteoarthritis, where inflammation and oxidative-stress-caused symptoms coexist with chondrodegenerative symptoms. Both sets of symptoms are caused by the destructive effects of free radicals (ROS and NO), proinflammatory cytokines (e.g., IL-1β and TNFα) and MMPs, all of which are produced in excess by various joint cells in patients suffering from osteoarthritis. A methylthiazolyldiphenyl-tetrazolium bromide (MTT) fluorogenic assay was used to quantify cell viability. Test compounds **1**–**3** did not affect the ability of chondrocytes to metabolize tetrazolium salts, demonstrating that these compounds do not interfere with cell viability (data not shown).

In consideration of the significant properties shown by compound **1**, molecular docking simulations were performed to probe the probable interactions between the MMP-13 enzyme, implicated in osteoarthritic pathology, and compounds **1**–**3**. There are a number of published crystal structures of MMP-13 in complex with competitive, zinc-chelating inhibitors or highly potent, noncompetitive (allosteric), non-zinc-chelating inhibitors.[3,8] Two structures, representing the two classes of inhibitors, were selected from the Protein Data Bank (PDB) as templates: 3KRY[8] (1.90 Å) and 3KEK[3b] (1.97 Å). These two conformations were selected as targets for docking studies. Docking calculations were performed using FlexX (FlexX Release 3.1, BiosolveIT GmbH, Sankt Augustin, Germany, 2009; http://www.biosolveit.de) on models constructed using the Molecular Operating Environment software (MOE 2010.10, Chemical Computing Group, Montreal, QC, Canada, 2010; http://www.chemcomp.com), used also for the calculation of van der Waals interactions and the preparation of enzyme and molecule structures, and Maestro (Maestro version 9.1, Schrödinger LLC, New York, NY, USA, 2010; http://www.schrodinger.com), to confirm, evaluate and analyze the docking poses obtained.

The binding site of MMP-13 in the open S1′ conformation (seen in both 3KRY[8] and of 3KEK[3b]) was analyzed to evaluate its suitability for docking experiments. In both complexes, the amino acid sequences and their spatial dispositions are very similar (RMSD = 0.740 Å). The type of co-crystallized ligand, zinc-chelating or non-zinc-chelating (allosteric), gave us important information on which residues are involved in both situations. The essential residues involved in allosteric binding to MMP-13 (PDB: 3KEK[3b]) are His 222, Thr 245, Leu 218, Thr 247, Asn 215 and Lys 140 along with many water molecules, while the amino acids involved in the binding of zinc-chelating inhibitors (3KRY[8]) are Tyr 244, Leu 185, Ala 186 along with coordination with the zinc atom. This should permit the evaluation of the possible binding modes of compounds **1**–**3**. Compound **1** seems to possess the required chemical functionalities for binding deeply in the active site. In a preliminary docking experiment, **1** seemed to stably bind in the enzymatic pocket. Superimposition and docking using the 3KEK[3b] structure showed that by gradually enlarging the levels of freedom of amino acids residues in the binding site, variation in the RMSD threshold value with initial conformation is quite small (0.128 Å for 3KEK[3b]; 0.134 Å for 3KRY[8]; the results are average for all conformations obtained by docking simulations).

Docking studies indicate that compound **1** does not coordinate the zinc atom, but strongly interacts with His 222, Thr 245, Leu 218 and Ala 238 (Figure 2). In particular, the thiazolidine moiety interacts with Thr 245 and a water molecule. The benzisothiazole moiety interacts with Pro 255 and Lys 249. In some particular configurations, the molecule becomes inverted in the binding site and the benzisothiazole fragment interacts with Tyr 244 and Thr 245. The methoxyphenyl group is predicted to have a stable interaction with His 222 and water molecules. From molecular dynamics experiments, we noted that interaction of compound **1** within the binding channel is pre-

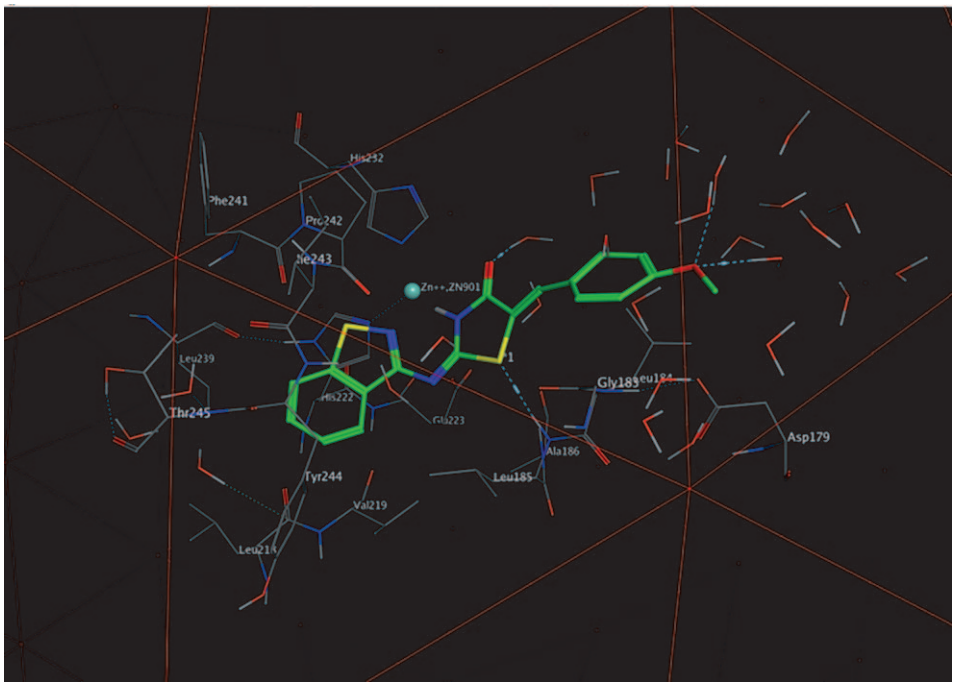

Figure 2. Model of compound **1** docked into PDB structure 3KEK.[3b]

dicted to be very stable. Moreover, the distance of compound **1** (chelating portion) from the Zn^{2+} cation is 9.7 Å on average. The results obtained by docking experiments using the enzyme structure 3KRY[8] predict that the distance between compound **1** and the zinc atom is too great for a close interaction (~7.50 Å). The amino acids involved in this case are Tyr244 and Leu184, and we also noted possible coordination with water molecules (Figure 3). In docking studies using the *meta* and *ortho* analogues **2** and **3**, respectively, the compounds failed to bind deeply in the binding site, probably because their calculated minimum energy conformation is strongly bent. The docking results obtained with both crystal structures give similar conclusions. This seems to validate the approach and make further studies possible.

In summary, the studies presented here describe our attempts to understand how a novel benzisothiazolylliminothiazolidin-4-one, endowed with potent antidegenerative activity on cartilage and inhibitory activity against a number of inflammation mediators, binds the zinc endopeptidase MMP-13. A model of the non-zinc-chelating binding mode is presented. These results further support the potential of compound **1** as a lead in the search for effective MMP-13 inhibitors and suggest optimization for the design of pharmacologically active agents to treat osteoarthritis. As the mechanism of cartilage degradation is multifactorial, it is essential to tackle this orthopedic disease with treatments that not only protect the cartilage against degenerative damage by stimulating the intrinsic repair capacity of chondroprotection, but that also neutralize the inflammatory/destructive potential of mediators involved in inflammatory/oxidative stress, such as nitric oxide, free radicals and inflammatory cytokines. Heteroarylimino-4-thiazolidinones **1**–**3** can be considered to be lead compounds for the development

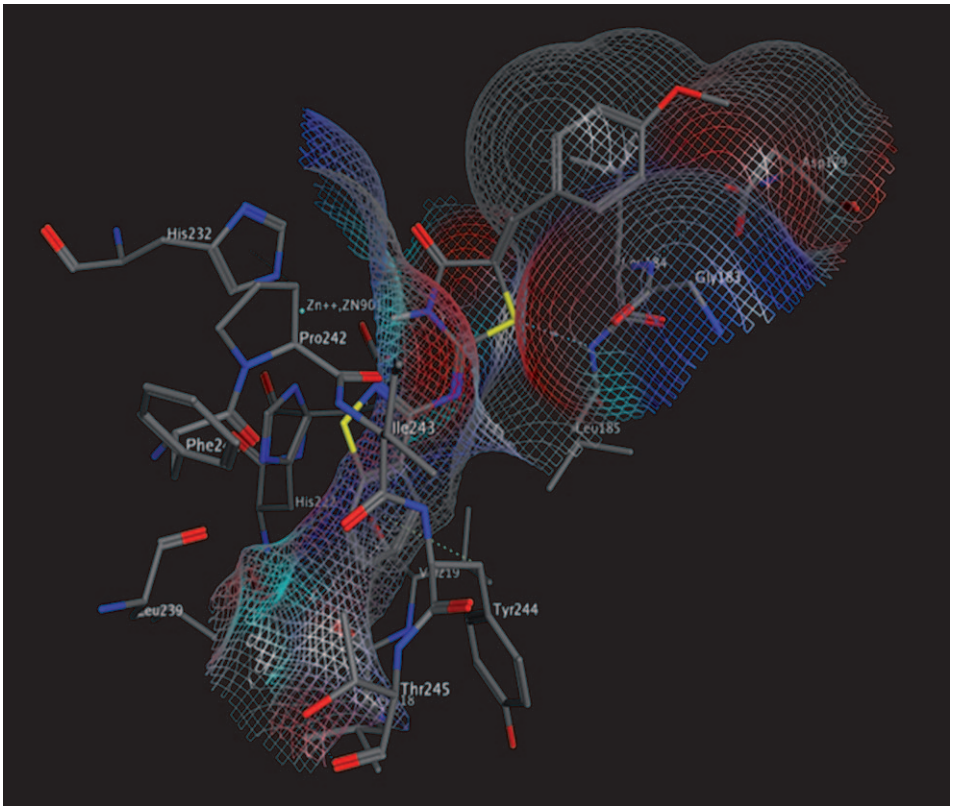

Figure 3. Model of compound **1** docked into PDB structure 3KEK[3b] and representation of van der Waals contour. Water molecules and the hydrogen atoms have been omitted for clarity.

of novel inhibitors of cartilage degradation for the treatment of osteoarthritis.

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