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Amidinobenzisothiazole derivatives with antidegenerative activity on cartilage

Annamaria Panico [a,*], Paola Vicini [b], Matteo Incerti [b], Venera Cardile [c], Barbara Gentile [a], Giuseppe Ronsisvalle [a]

[a] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Catania, V.le A. Doria 6, 95125 Catania, Italy*
[b] *Pharmaceutical Department, University of Parma, Parco Area delle Scienze 27/A, 43100 Parma, Italy*
[c] *Department of Physiological Sciences, University of Catania, V.le A. Doria 6, 95125 Catania, Italy*

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Abstract

N-(Benzo[*d*]isothiazol-3-yl)amidines were synthesised and evaluated for their antiinflammatory activity. Encouraging results led us to evaluate these derivatives on the prevention of cartilage destruction in articular disease. Antidegenerative activity was assayed on culture of porcine nasal cartilage and diarthroidal joint human cartilage in the presence of interleukin-1β (IL-1β). The amount of glycosaminoglycans (GAGs) and the production of nitric oxide (NO) in the culture medium were determined. The obtained results showed that all the compounds, in the presence of IL-1β, blocked the cartilage breakdown, with different behaviour. The antidegenerative activity is more evident in human cartilage.

Keywords: *N*-(Benzo[*d*]isothiazol-3-yl)amidines; Cartilage; IL-1β; NO; GAGs

1. Introduction

Benzo[*d*]isothiazole is a bicyclic system occurring in various molecules endowed with biological activity as, for example, antiinflammatory activity [1–4]. Our interest in this field led us to synthesise benzo[*d*]isothiazole derivatives bearing an amidino moiety, the non-acidic isosteric nitrogen analogue of the carboxylic group, with the aim to study their potential effect as antiinflammatory agents. In our previous studies encouraging results emerged from in vivo antiinflammatory tests of some benzo[*d*]isothiazolylamidines, but nothing was evaluated in vitro and, to our knowledge, only a few data are reported regarding pharmacological aspects of benzisothiazolyl derivatives endowed with antiinflammatory activity [5,6].

Concerning antiinflammatory activity, it has been observed that some NSAIDs, in spite of their effectiveness in suppressing inflammation and pain, may themselves potentially compromise the tissue metabolism of cartilage, thereby possibly abrogating the long term benefit of suppression of inflammation [7–10]. Thus modern pharmacology is interested in the development of new antiinflammatory compounds endowed with antidegenerative activity on cartilage, during inflammatory process [11].

Nitric oxide (NO) and glycosaminoglycans (GAGs) can be considered key molecules of cartilage destruction. In the case of inflammatory disease, GAGs release is a consequence of increased matrix protease activity leading to the cleavage of collagen and proteoglycans, fundamental constituents of cartilage. Moreover, the catabolic effect of NO determines the inhibition of proteoglycan synthesis and stimulates the condrocyte production of proenzymes that, converted into active enzymes (metalloproteinases), causes cartilage breakdown [12–14].

NO is a very small and ubiquitous molecule synthesised from L-arginine by NO synthase (NOS). NO is produced in the joint by chondrocytes, synoviocytes and osteoblasts and is released at high extent during inflammatory process. Endogenous NO has been

* Corresponding author.
E-mail address: panico@mbox.unict.it (A.M. Panico).

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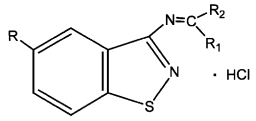
Table 1
Amidinobenzisothiazole derivatives under study



Compound	R	R_1	R_2
1	H	NH_2	CH_3
2	CH_3	NH_2	CH_3
3	H	$N(CH_3)_2$	H
4	CH_3	$N(CH_3)_2$	H
5	H	NH_2	C_6H_5
6	CH_3	NH_2	CH_2
			$-CH=CH_2$
7	H	NH_2	2-pyridyl

described as a proinflammatory molecule showing interaction with cytokines and inflammatory products of the cyclooxygenase pathway. Several studies have shown that NO increases production of interleukin-1β (IL-1β) and eicosanoid products, which may result in an exacerbated inflammatory response [15–17].

From the above considerations, in the present work we decided to investigate the effects on NO production and GAGs release of a selected number of *N*-(benzo[*d*]isothiazol-3-yl)amidines synthesised by us (Table 1), previously demonstrated to possess antiphlogistic–analgesic activity [5,6].

Their in vitro effects were evaluated in this study, on the metabolism of porcine nasal cartilage and human cartilage treated with IL-1β, a cytokine released during inflammatory process. Parallel experiments were also carried out in the presence of indomethacin as reference drug.

2. Experimental

2.1. Chemistry

The *N*-(benzo[*d*]isothiazol-3-yl)amidines **1**–**7** (Table 1) selected for this study, were prepared following the methods previously described by Vicini et al. [5,6], through nucleophilic addition of the appropriate 3-aminobenzisothiazole to the carbon of the selected cyanides (**1**, **2**, **5**–**7**), or by reacting the appropriate 3-aminobenzisothiazole with *N*,*N*-dimethylformamide dimethyl acetate (**3**, **4**). Analytical data (m.p., NMR, IR) and more detailed procedures for the synthesis of starting, intermediate and final compounds are described in Refs. [5,6].

The *N*-(benzo[*d*]isothiazol-3-yl)amidines under study were assayed as hydrochlorides dissolved in DMSO.

2.2. Biological evaluation

2.2.1. Porcine nasal 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) which were placed into 24-well plates, each containing 1 ml of Dulbecco's modified eagles medium (DMEM, Sigma) phenol red free, 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) IL-1β (10 ng/ml); (c) compounds (1–10–100 μg/ml) combined with IL-1β (10 ng/ml), and indomethacin (10^{-5} M). After 120 h the supernatants of cartilage culture were collected for different assays [18,19].

2.2.2. Human articular cartilage culture

The human articular cartilage deriving from operating pieces, in fragments of small dimensions, was washed in Hank's balanced salt solution containing antibiotic (penicillin 100 U/ml, streptomycin 100 μg/ml). The fragments were set in a sterile plate, into 24 wells each containing 1 ml of DMEM (Sigma) phenol red free, 10% foetal bovine serum (FCS), 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 2.5 mg/ml of amphotericyn B. After 24 h in an incubator at 37 °C, with 5% of CO_2 and 95% of humidified air, the media were removed and cartilage samples were treated as follows ($n = 4$ per group): (a) control medium; (b) IL-1β (10 ng/ml); (c) compounds (1–10–100 μg/ml) combined with IL-1β (10 ng/ml), and indomethacin (10^{-5} M). After 120 h the supernatants of cartilage culture were collected for different assays [18,19].

2.2.3. Determination of nitrite levels

Nitrite levels were determined in the culture media using the Griess reaction [18]. To single samples (100 μl), placed into 96 wells microplate, 100 μl of sulphanilamide (1% w/v) were added. The plate was wrapped in aluminium foil, and shaken briefly. *N*-(1-Naphthyl)ethylenediamine (100 μl) was added to each sample, shaken briefly, wrapped in 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 $NaNO_2$ standard curve (0–120 μM).

2.2.4. Determination of GAGs

GAGs were quantified using 1,9-dimethyl methylene blue (DMB) as previously reported [19,20]. A standard

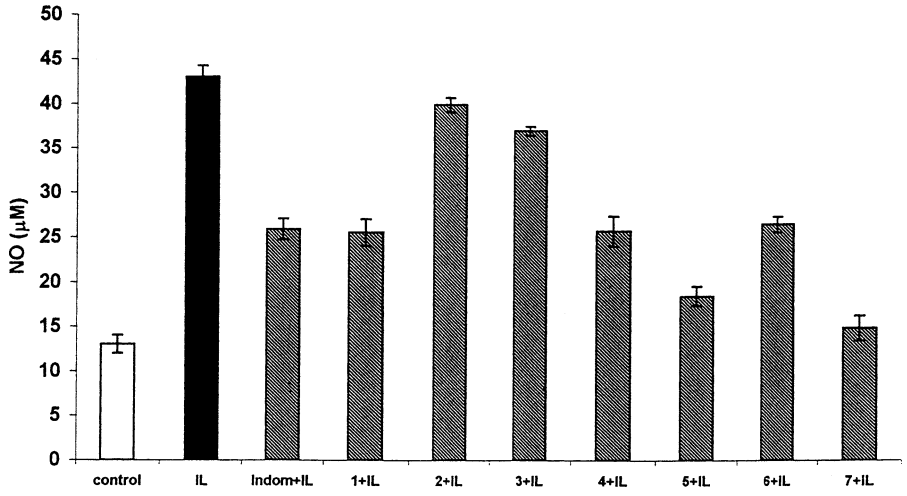


Fig. 1. NO production (means ± SEM) from porcine nasal cartilage into the culture medium 120 h after the addition of amidinobenzisothiazole derivatives **1**–**7** (100 μg/ml) or indomethacin with IL-1β. Values are expressed as μM.

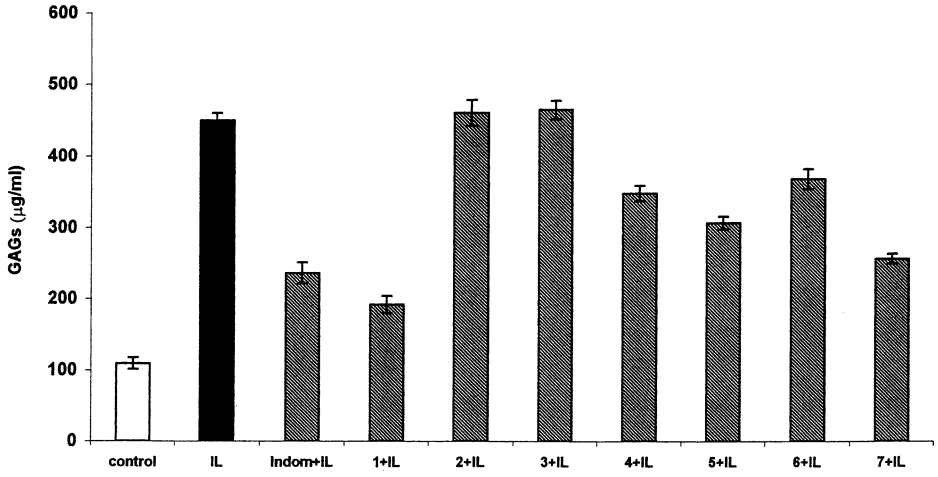


Fig. 2. GAGs release (means ± SEM) from porcine nasal cartilage into the culture medium 120 h after adding of amidinobenzisothiazole derivatives **1**–**7** (100 μg/ml) or indomethacin with IL-1β. Values are expressed as μg/ml.

curve was used for determination of GAG concentration (100–500 μg/ml). The standard GAG used is chondroitin sulphate C, derived from shark cartilage. The absorbance was measured at $\lambda = 535$ nm.

2.2.5. Statistical analysis

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

3. Results and discussion

The *N*-(benzo[*d*]isothiazol-3-yl)amidines **1**–**7**, tested for their antidegenerative effect at different concentrations (1–10–100 μg/ml), showed dose-dependent activity (data not shown) both in porcine and in human cartilage, in our experimental models. Figs. 1 and 2 report the results obtained after 120 h at the concentration of 100 μg/ml in porcine cartilage, on NO and GAGs release, respectively.

The effects of compounds **1**, **5** and **7** (resulted the most active with porcine cartilage), on NO and GAGs release using slices of human articular cartilage, are reported in Figs. 3 and 4, respectively.

Tissue samples of porcine and human articular cartilage were treated, for the present study, with compounds **1**–**7** combined with IL-1β. This cytokine was used to simulate the inflammatory process. It is in fact well known that during the inflammatory processes, accompanying inflammatory disease, monocytes and macrophages release IL-1β that was shown to reduce proteoglycan synthesis by articular chondrocytes and to

increase the degradation of these macromolecules [21]. This effect was also confirmed by our experimental results, as can be observed in Figs. 1–4 by comparing, with control data, the remarkable increased release of NO and GAGs, after treatment with IL-1β at concentration of 10 ng/ml. The controls (containing only DMSO, the solvent used to dissolve compounds) produced a very low amount of NO mainly due to the constitutive NOS and consequently a low amount of GAGs. It is worth noting that the NO level and the GAGs release in human cartilage before (control data) and after treatment with IL-1β were lower with respect to the values obtained in porcine cartilage, but, as previously reminded, they showed a similar dose-dependent activity. This might be supported by differences of the source and physical structure of the used cartilage [22]. Indomethacin was used as reference drug and samples treated with indomethacin combined with IL-1β showed a significant decrease ($P < 0.01$), relatively to the IL-1β treated samples, both on NO and GAGs release. However, these latter responses were significantly higher than in each respective untreated control.

As shown in Fig. 1, all the tested compounds **1**–**7** when combined with IL-1β exhibited a significant reduction of NO release compared to the samples treated with IL-1β. The effect of compounds **5** (18.45 ± 0.9) and **7** (14.83 ± 1.2) was higher than that observed with indomethacin and IL-1β (25.86 ± 0.86), while compounds **1**, **4** and **6** had a similar behaviour to indomethacin. Concerning the activity on GAGs release (Fig. 2), compounds **1**, **4**, **5**, **6** and **7**, combined with IL-1β, showed an inhibitory effect by comparing the samples treated with IL-1β. Only compound **1** exhibited a significant higher effect (191.54 ± 11) than that observed when the samples were treated with indomethacin (236.08 ± 10).

In the present work, we also compared the activity of these compounds on porcine and human cartilage. The comparison of biological response in these systems, for NO production and GAGs release permitted us to observe a better behaviour for human cartilage. As it is shown in Fig. 3, we have for NO an inhibition %,

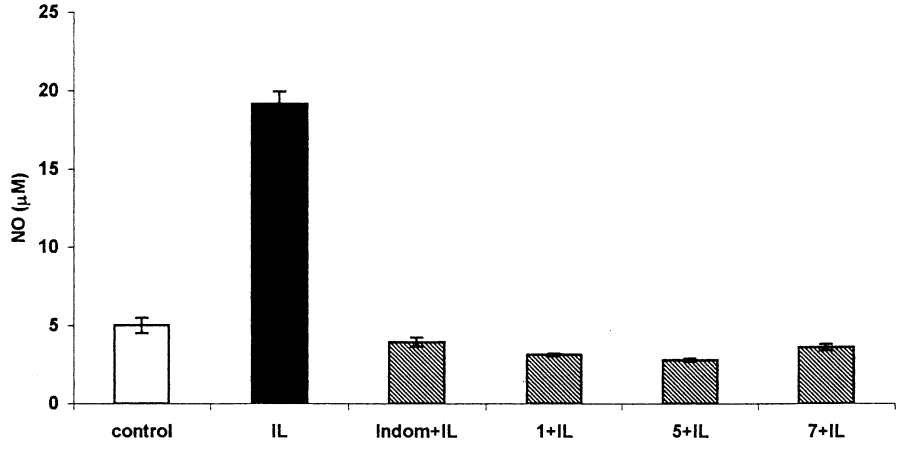


Fig. 3. NO production (means ± SEM) from human cartilage into the culture medium 120 h after the addition of amidinobenzisothiazole derivatives **1**, **5**, **7** (100 μg/ml) or indomethacin with IL-1β. Values are expressed as μM.

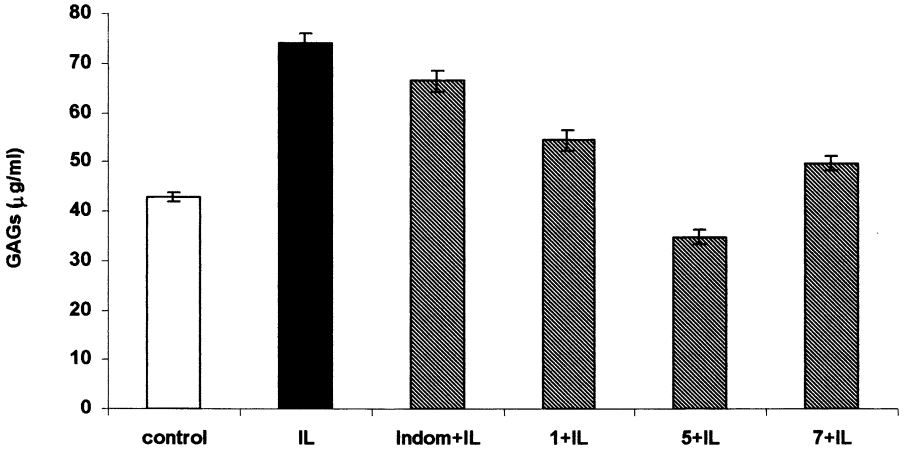


Fig. 4. GAGs release (means ± SEM) from human cartilage into the culture medium 120 h after adding of amidinobenzisothiazole derivatives **1**, **5**, **7** (100 μg/ml) or indomethacin with IL-1β. Values are expressed as μg/ml.

compared to the samples treated with IL-1β, of 83.7 for compound **1** and of 85.3 for compound **5**, higher than that observed in the samples treated with indomethacin and IL-1β (79.65), while compound **7** (inhibition % = 81.2) has a similar behaviour to indomethacin. In regard, the inhibitory effect of these compounds on GAGs release (Fig. 4), it is possible to observe the higher inhibitory effect for compounds **1**, **5**, **7** combined with IL-1β than for the samples treated with indomethacin and IL-1β. The compound **5** exhibited the highest activity. From a structural point of view the most effective *N*-(benzo[*d*]isothiazol-3-yl)amidines **1**, **5** and **7** in preventing IL-1β harmful effects on cartilage are characterised by a hydrogen as R, while different substituents (methyl, phenyl, pyridyl) are allowed as R_2. An unsubstituted amino group as R_1 seems necessary for this type of pharmacological activity, as *N*,*N*-dimethylformamidines **3** and **4** showed very slight or no effect on the NO and GAGs release. It is worthwhile noting that *N*-(benzo[*d*]isothiazol-3-yl)acetamidine (**1**) exhibited the most potent action, in porcine cartilage, on GAGs release, but it was not the most active compound on NO release inhibition. *N*-(Benzo[*d*]isothiazol-3-yl)benzamidine (**5**) is clearly the most effective both in NO and GAGs release in human cartilage. This suggests different mechanisms for the *N*-(benzo[*d*]isothiazol-3-yl)amidines resulted active in this study.

In conclusion, the insertion of selected substituents in the benzisothiazolylamidine system can supply compounds able to block the cartilage destruction during the inflammatory process as simulated in our experimental model and further investigations are warranted in order to reach a full understanding of the structure–activity relationship in this very promising class of antiinflammatory–antidegenerative compounds.

Acknowledgements

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