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Can Non-steroidal Anti-inflammatory Drugs Act as Metalloproteinase Modulators? An In-vitro Study of Inhibition of Collagenase Activity

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Abstract

The in-vitro effects of some non-steroidal anti-inflammatory drugs and some analgesic drugs on collagenase activity were studied by use of a self-quenched fluorogenic esapeptide as substrate. The increased fluorescence signal arising as a result of peptide cleavage by collagenase was recorded and related to the inhibitory potency of the drugs. The effective concentrations in collagenase modulation were also correlated with the levels of the drugs in the plasma and synovial fluids of patients receiving therapeutic doses.

Six of the tested drugs, nimesulide, piroxicam, tolmetin, meloxicam, sulindac and sodium meclofenamate, inhibited enzyme activity with IC50 values (concentrations resulting in 50% inhibition) ranging from 1·9 to 28·2 μM and K_i (apparent inhibition constant) ranging from 0·83 to 21·8 μM. Much of the activity was restored after dialysis of the enzyme–drug complex, demonstrating the reversibility of the effect.

Although these results indicate that some anti-inflammatory drugs could modulate enzymatic activity involved in the degradation of the extracellular matrix, their possible pharmacological involvement as collagenase inhibitors in collagen degradative diseases remains to be assessed by clinical studies.

Cartilage integrity requires a balance between synthesis and degradation of matrix components; this degradation is increased in rheumatic diseases through the action of chondrocyte-derived metalloproteases (Walakovitis et al 1992). Matrix metalloproteases (MMP), such as collagenase and stromelysin, play important roles in degrading collagen and proteoglycan in the extracellular matrix seen in rheumatoid arthritis and osteoarthritis. Increased collagenase activity has been identified in-situ in osteoarthritic cartilage in man and in the experimental dog model of osteoarthritis (Pelletier et al 1983a). Furthermore, the collagenase level has been correlated with the severity of cartilage lesions (Pelletier et al 1983b). The alterations in the collagen and proteoglycan balance in arthritic cartilage suggest increased proteolytic breakdown of these molecules and, even if a natural endogenous inhibitor of metalloproteases (TIMP) is present in articular cartilage, its levels do not increase to the same extent as those of the degrading enzymes in some pathological conditions (Dean 1991). Thus, the use of synthetic MMP inhibitors as drugs for future prevention of connective tissue breakdown is promising (Cawston 1996). Moreover, it will be interesting to evaluate if the blocking of one MMP is sufficient to halt the progressive and chronic destruction of connective tissue found in the arthritides, or if it will be necessary to combine proteinase inhibitors, either in sequence or with other exogenous agents.

Because metalloprotease activity is the final result of enzyme synthesis, activation, and regulation, the observed suppressive effects of enzyme activity exerted by some drugs cannot yet be explained. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat patients with osteoarthritis or rheumatoid arthritis but their effect on articular cartilage metabolism during the course of these rheumatic diseases in man remains a subject of debate. It is believed that some NSAIDs (i.e.

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salicylates and indomethacin) accelerate cartilage destruction by impairing proteoglycan synthesis by chondrocytes, whereas others have a chondroprotective effect (Brandt 1987; Rashad et al 1989; Vignon et al 1990). Such studies have yielded conflicting data, perhaps reflecting differences in NSAID classes, methodology or source (type, kind) of cartilage used. Although there is a large volume of literature about therapeutic uses of NSAIDs in these diseases, few studies have provided data on the action of these compounds on cartilage metabolism in man (Brandt 1987; Rashad et al 1989).

The aim of this study was to evaluate the in-vitro effects of several classes of NSAID (nimesulide, piroxicam, meloxicam, tolmetin, sodium meclofenamate, sulindac and indomethacin) and of some analgesic drugs (morphine and paracetamol) on enzyme activity of collagenase type XI from *Clostridium hystolyticum*. We have also tested our system on synovial fluids from patients with inflammatory or degenerative diseases. Fluorimetric assays were performed using a synthetic esapeptide as substrate (Knight et al 1992), and the increased fluorescence signal as a result of peptide cleavage by collagenase was recorded (Barrett et al 1989; Di Giulio et al 1996). By use of this test the inhibitory potency of the compounds cited above was studied. The inhibitory and inactivating behaviour of the drugs was also evaluated on the basis of the capacity of the enzyme to recover its activity after extensive dialysis.

Our results indicate that some of the above mentioned anti-inflammatory molecules could act as modulators of the inflammatory processes characterized by collagen breakdown. The concentrations of the NSAIDs effective in the modulation of collagenase activity seem to correlate with the levels of the same drugs found in the plasma and synovial fluids of patients receiving normal therapeutic dosages.

Materials and Methods

Chemicals and instrumentation

Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–NH_2 (Mca substrate) and Mca–Pro–Leu–Gly (standard) were purchased from The Peptide Institute (Osaka, Japan). Collagenase type XI from *Clostridium histolyticum* (4·0 units (mg solid)$^{-1}$), hyaluronidase type III from sheep testes and polyoxethylene 23 lauryl ether (Brij 35) were from Sigma (St Louis, MO). Tris (hydroxymethyl)aminomethane was from Fluka Chemie (Buchs, Switzerland), dimethylsulphoxide (DMSO) and all other chemicals, of reagent grade, were from Merck (Darmstadt, Germany). The NSAIDs were kindly provided by the respective manufacturers: indomethacin (mol. wt = 357·81; Sigma–Tau, Italy), sodium meclofenamate (336·1; Parke–Davis, Italy), tolmetin sodium dihydrate (315·3; Janssen Cilag, Italy), piroxicam (331·4; Pfizer, Italy), sulindac (356·4; Merck Sharpe & Dohme, Italy), paracetamol (151·2; Angelini Ricerche, Italy), morphine (375·86; Carlo Erba, Italy), nimesulide (308·3; LPB–Sandoz, Italy), and meloxicam (351·4; Boehringer Ingelheim, Italy).

Experiments were performed with a Perkin–Elmer LS-50B luminescence spectrophotometer, computer controlled by Fluorescence Data Manager Software (Perkin–Elmer, Bucks, UK). The excitation and emission wavelengths were 328 nm (slit 2·5 nm) and 393 nm (slit 5·0 nm), respectively.

All experiments were performed in 0·1 M Tris–HCl buffer, pH 7·5, containing 0·1 M NaCl, 10 mM $CaCl_2$, 0·05% Brij 35 and 0·02% NaN_3, referred to in the text as Tris buffer.

Standard curve

The reference curve was constructed by use of a standard solution obtained by dissolving Mca–Pro–Leu–Gly (MW 501·54, 0·11 mg) in dimethylsulphoxide (DMSO; 0·1 mM stock solution; 2·2 mL). The solution was diluted directly in the cuvette with Tris buffer to a final concentration ranging from 7·8 to 125 nM (i.e. from 23·4 to 375·0 pmol) of the reference compound. Three independent determinations were performed on each diluted solution and the mean ± s.d. of the values were used to construct a standard curve (Di Giulio et al 1996) from which a 0·70 correlation factor was extrapolated and used to calculate the amount (pmol) of hydrolysed substrate from the emission fluorescence units recorded.

Enzyme activity measurements

The Mca substrate was dissolved in DMSO at a concentration of 500 μM and stored frozen. This stock solution was diluted with Tris buffer to a final concentration of 5 μM. The *Clostridium hystolyticum* collagenase solution (4·0 units mL^{-1}) in Tris buffer was freshly prepared. The 10^{-3} M stock solution of the inhibitors were prepared by adding DMSO to the weighed powder (100 μL mg^{-1}) and diluting with Tris buffer. No turbidity was observed in the solution and we proved experimentally that 0·1% (v/v) DMSO in the sample mixtures did not affect collagenase activity.

Experiments were performed by incubating diluted substrate (100 μL; final concentration in cuvette 167 nM) with the enzyme solution (100 μL, 0·4 units) in Tris buffer for 5 min at 20°C. Cleavage of the peptide by collagenase (Barrett et al 1989)

removes the internal quencher of the probe making the product highly fluorescent. In the inhibition tests different concentrations of the drugs were also present. After incubation the fluorescence signal was monitored by recording the signal for 3 min.

The extent of substrate hydrolysis was calculated by multiplying the fluorescence values by the factor 0·70, derived as discussed above. The K_m (Michaelis constant) value was determined for a substrate-concentration range of 40–350 nM and calculated by means of a Lineweaver–Burke double-reciprocal plot. The inhibition of collagenase activity by each compound was tested at fixed inhibitor concentration as reported in Table 1. The IC50 values (concentrations resulting in 50% inhibition) were determined by plotting residual enzyme activity against drug concentrations and extrapolated graphically. The apparent inhibition constants (K_i) were also calculated assuming a competitive inhibition model and applying the equation:

$$V_0/V_i = 1 + K_m[I]/\{(K_m + [S])K_i\} \quad (1)$$

where: V_0 and V_i are, respectively, the initial hydrolysis rate of the Mca substrate in the absence and in the presence of inhibitors; [I] is the drug concentration and K_m and [S] are the Michaelis constant and substrate concentration. Plots of V_0/V_i against drug concentration yielded a line of slope $K_m/K_i(K_m + [S])$ (Figure 1). The inhibition parameters of the NSAIDs are reported in Table 2.

Reactivation experiments

Collagenase was incubated for 15 min at 20°C with two different concentrations of each active compound (the concentrations are given in Table 3) and the mixtures were divided into two portions. The first was immediately tested for inhibitory potency before dialysis; the second was dialysed for 24 h at 5°C against Tris buffer and then measured to test the reversibility effect. The pre- and post-dialysis tests were performed using a constant substrate concentration of 167 nM.

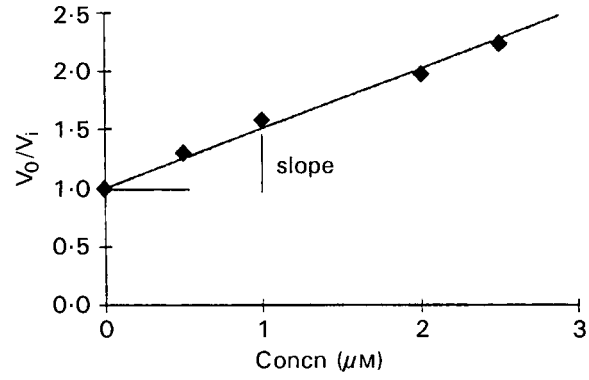

Figure 1. Plot of the V_0/V_i (the ratio of the initial hydrolysis rate of the drug in the absence of nimesulide to that in its presence at different concentrations) against nimesulide concentration. Each point is the mean of results from three determinations (s.d. < 6%). The slope was extrapolated graphically as shown and used to determine K_i. The curve was obtained by linear regression analysis.

Time-course inactivation studies

Inactivation experiments were performed by incubating enzyme (0·40 units) and the selected compound at concentrations near the IC50 value at 20°C in Tris HCl buffer (0·1 M, pH 7·5) as previously reported. At different times, 150 μL samples of this mixture were withdrawn and the reaction was started by adding the substrate (167 nM) to a final volume of 3 mL. If progressive inhibition of collagenase activity was detected, the compound was considered an inactivator.

Clinical specimens

Synovial fluid was obtained from 13 patients with rheumatoid arthritis and two with osteoarthritis. At the time of arthrocentesis the patients were under

Table 1. Inhibition (%) of collagenase activity upon incubation with drug (10 μM).

Drug	Inhibition
Paracetamol	4·6 ± 5·6
Tolmetin	20·2 ± 2·7
Sulindac	28·7 ± 5·2
Nimesulide	91·9 ± 2·0
Morphine	6·7 ± 6·6
Meloxicam	40·6 ± 0·7
Indomethacin	5·6 ± 1·4
Piroxicam	35·0 ± 3·3
Meclofenamate	19·1 ± 2·1
Ascorbic acid	3·4 ± 2·7

Values are means ± s.d. of results from six independent experiments.

Table 2. Inhibition of collagenase activity by NSAIDs.

Drug	In-vitro inhibition of collagenase activity	IC50 (μM)	K_i (μM)
Nimesulide	+	1·9	0·83
Piroxicam	+	13·6	7·90
Tolmetin	+	25·6	21·30
Meloxicam	+	14·8	8·53
Sulindac	+	28·2	15·70
Meclofenamate	+	26·8	21·80

Assays were performed at 20°C in Tris buffer, pH 7·5, using fixed concentrations of collagenase (0·4 units mL^{-1}) and Mca substrate (167 nM). For graphical determination of IC50 and K_i values (Figure 2) the compounds were tested in triplicate at each of four different concentrations.

Table 3. Restoration of collagenase activity by dialysis.

Drug	A (μM)	Collagenase inhibition		B (μM)	Collagenase inhibition	
		Pre-dialysis	Post-dialysis		Pre-dialysis	Post-dialysis
Tolmetin	25	64·9 ± 1·0	8·7 ± 4·1	70	91·6 ± 1·2	24·1 ± 3·8
Sulindac	30	67·9 ± 0·8	8·7 ± 2·6	50	81·8 ± 0·3	39·9 ± 1·5
Nimesulide	2	61·9 ± 8·2	10·6 ± 4·1	5	79·8 ± 4·0	37·8 ± 7·8
Meloxicam	15	39·2 ± 6·9	1·0 ± 0·5	40	77·4 ± 3·5	4·1 ± 3·7
Piroxicam	15	56·9 ± 0·3	5·4 ± 1·2	30	75·3 ± 0·9	17·0 ± 0·6
Meclophenamate	25	60·0 ± 1·5	12·1 ± 0·8	100	89·6 ± 0·3	6·1 ± 2·8

The dialysis was initiated 15 min after addition of the inhibitors, at concentrations close to their IC50 (A) or IC80 (B) values, to collagenase type XI solution. Dialysis was performed at 4°C against Tris buffer. Enzyme activity is expressed as percentage of control activity (i.e. collagenase incubated under the same conditions as the samples, but in the absence of the inhibitors). Data are means ± s.d. of results from six separate experiments. Comparison of the percentage inhibition pre- and post-dialysis (Student's *t*-test) showed that in all cases $P < 0.01$.

different therapeutic regimens. Synovial fluid was obtained from knee-joint effusions. After arthrocentesis the synovial fluid was separated from cells and debris by centrifugation and then stored frozen (−70°C; 1-mL portions) until use (Clark et al 1993). After thawing, the synovial fluid was treated with hyaluronidase H-2251 (1 h at room temperature), centrifuged and used for the enzymatic and inhibition tests (described above) and for protein determination by a standard dye-binding assay.

Statistical analysis

Results are expressed as the mean ± s.e.m. of results from six experiments. The data were analysed statistically by Student's *t*-test, *P* values < 0·01 being regarded as indicative of significance. The K_m and the conversion factor for substrate analysis, and the concentration causing half-maximum inhibition, denoted K_i and IC50, respectively, were obtained by linear regression analysis of the appropriate plots.

Results

In-vitro inhibitory effect of NSAIDs on collagenase activity

Table 1 shows the amount (%) of inhibition of collagenase activity upon incubation with each drug (10 μM). It is evident that at the same concentration only six of the tested compounds, tolmetin, sulindac, nimesulide, meloxicam, piroxicam and sodium meclofenamate, were able to inhibit enzyme activity partially, although to different extents (i.e. from 20 to 90%).

Kinetic parameters of drug enzyme inhibition

The six active drugs were analysed for their inhibitory potency and the apparent inhibition constant (K_i) and the drug concentration inhibiting 50% of enzyme activity (IC50) were then calculated. For calculation of K_i a competitive model was assumed (Materials and Methods) and graphical computation was performed by plotting the ratio of the initial hydrolysis rate in the absence of drug (V_0) to that in the presence of drug (V_i) at different concentrations. The slope of this plot was used to determine K_i, as shown in Figure 1 for nimesulide. The estimated K_i values, ranging from 0·83 μM for nimesulide to 21·8 μM for sodium meclofenamate, are reported in Table 2. The IC50 concentration was determined by dose–response studies. Curve fitting was performed by assuming a linear relationship between response and drug concentration and the optimum fit obtained by minimizing the sum of the squared errors (not shown). By use of this approach we estimated IC50 values ranging from 1·9 μM for nimesulide to 28·2 μM for sulindac (Table 2).

Reactivation studies

The reactivation experiments showed that collagenase activity was recovered after prolonged (24 h) dialysis of the enzyme–inhibitor complex against Tris buffer. Each compound was tested at two different concentrations, the first close to its calculated IC50 and the second at concentrations inhibiting 80–90% of the initial activity. It should be emphasized the enzyme always recovered approximately 90% of its initial activity. When higher concentrations of inhibitors were used some activity was also restored after dialysis, although to a different extent. Restoration of activity was particularly high for sodium meclofenamate, meloxicam, piroxicam and tolmetin whereas when sulindac or nimesulide were used as inhibitors recovery was only partial (Table 3). Activity is expressed as percent of control activity (i.e. that measured when collagenase was incubated under

the same conditions as the samples but in the absence of drug) and data are means ± s.d. of results from six separate experiments.

Time course of inhibition effect

Inactivation experiments demonstrated that the enzyme was not inactivated by interaction with the six drugs.

Experiments on clinical specimens

A set of experiments was performed with synovial fluid (15 μL) from patients with osteoarthritis or rheumatoid arthritis (total proteins ranging from 0·41 to 1·99 mg). In this set of trials we checked the capacity of nimesulide and meloxicam to inhibit the activity of collagenase from man. Of fifteen clinical specimens only three cleaved our substrate after treatment with hyaluronidase (Clark et al 1993). The specific activities were 82·1 ± 7·2, 66·3 ± 7·3 and 12·1 ± 0·4 pmol substrate transformed min^{-1} $(mg\ protein)^{-1}$, respectively. This activity was reduced to 27·2 ± 1·5, 21·9 ± 1·0 and 5·4 ± 0·2, respectively, in the presence of 2 μM nimesulide (estimated IC50) or 39·1 ± 2·1, 31·1 ± 2·3 and 5·6 ± 0·5, respectively, in the presence of 15 μM meloxicam.

Discussion

In chronic diseases such as rheumatoid arthritis and osteoarthritis, an important stage of tissue injury is the irreversible degradation of connective tissue molecules such as collagen. The complex mechanism responsible for irreversible joint damage in many arthritic disorders remains unclear (Blackburn et al 1991). Therapeutic agents capable of inhibiting the activation of matrix metalloproteases (MMP) and reducing their activity might have a role in altering the course of tissue injury. Because MMP activity is a combination of the effects of enzyme synthesis, activation and inhibition, the suppressive effects on enzyme activity exerted by some therapeutic agents, e.g. NSAIDs, in-vivo cannot yet be explained (Vignon et al 1990). However, it is well known that these drugs can help reduce cartilage catabolism in patients with osteoarthritis (Vignon et al 1991) and the inhibition of chondrocyte-derived MMP by nimesulide has been reported (Goodman & Gilman 1996). Such an effect was apparent from our in-vitro experiment in which the activity of collagenase decreased by 20–90% when incubated with NSAIDs (10 μM, an amount within the range of the actual synovial fluid therapeutic concentration in patients with arthritic diseases) (Table 1). Other drugs such as morphine, paracetamol, indomethacin and ascorbic acid tested under the same conditions did not affect collagenase activity. Although the extrapolation of our data to in-vivo conditions might be the subject of debate, the inhibitory effect on collagenase of these drugs, at concentrations achievable in the synovial fluid of patients receiving long-term therapy (Table 4), might be an interesting finding. Furthermore, because the calculated in-vitro IC50 for nimesulide and tolmetin was one sixth the reported concentration of these drugs in synovial fluid, it can be hypothesized that these drugs help reduce abnormal catabolism of cartilage in rheumatic patients. A similar trend in-vitro was also observed for sulindac, meclofenamate and meloxicam, although these drugs inhibited bacterial collagenase only at supratherapeutic concentrations (i.e. the respective IC50 and K_i values were higher than their concentrations in synovial fluid, Table 4). It should be emphasized that sulindac is the prodrug of a more active sulphide metabolite which has two to five times the pharmacological activity of the parent compound (Ravis et al 1993). Also noteworthy is the behaviour of piroxicam, for which the IC50 is twice the therapeutic synovial fluid concentration but which, because of its high elimination half-life, probably exerts a more durable inhibitory effect on collagenase activity.

As the mechanism of this inhibition we initially hypothesized chelation with the Zn^{2+} of the MMP collagenase, but EDTA, a well known chelating agent, did not inhibit enzyme activity under the same conditions. Inactivation experiments clearly demonstrated that no time-course inactivation occurred upon incubation of the collagenase enzyme with the specific drug, which excludes the possibility that inhibition by NSAIDs is based on the mechanism of action of collagenase. Other studies have demonstrated that collagenase activity is inhibited by chemotherapeutic agents including tetracyclines (IC50 15–350 μM; Golub et al 1991), anthracyclines (IC50 37–90 μM; Karakiulakis et al 1990) and rifamycines (IC50 13–21 μM; Di Giulio et al 1996). However, these IC50 values are at least an order of magnitude higher than those of nimesulide.

For all the drugs, removal by dialysis of concentrations close to the respective IC50 led to recovery of the potency of the enzyme. For higher drug concentrations, however, enzyme treated with nimesulide and sulindac recovered only partially. This observation could be of particular therapeutic relevance to the intra-articular administration of the two drugs. This route, in fact, enables achievement of higher drug concentrations, which, in turn,

Table 4. In-vitro anti-collagenase effect and effective concentrations of analgesic and non-steroidal anti-inflammatory drugs.

Drug	IC50 ($mg\,L^{-1}$)	Therapeutic plasma concentration ($mg\,L^{-1}$)	Therapeutic synovial fluid concentration ($mg\,L^{-1}$)	Vd ($L\,kg^{-1}$)	$t_{1/2}$(h)
Nimesulide	0·59	3·0[a]	1·38–2·39[b]	0·19–0·39[c]	3§
Tolmetin	8·07	45·4[e]	5·6[e]	0·54 ± 0·07[f]	5§
Piroxicam	4·51	4·45–8·02[g]	2·0[h]	10·0[i]	50§
Sulindac	10·05	5·0[j]	–	2[k]	7§
Meclofenamate	9·01	1·8–7·2[l]	1·8–7·2[l]	23·3[l]	2–4§
Meloxicam	5·20	1·0–2·0[m]	0·5–1·0[m]	10·7[n]	20§§§
Indomethacin	13·84	0·3[g]	0·3[d]	0·29 ± 0·04[o]	3§
Morphine	>37·58	$65·8 \times 10^{-3}$ [d]	–	3·3 ± 0·9[p]	3§
Paracetamol	>15·12	7·5[g]	–	0·95 ± 0·12[q]	2§

The IC50 is the amount of drug inhibiting in-vitro collagenase activity by 50%, Vd is the volume of distribution, and $t_{1/2}$ is the elimination half-life. [a]Pelletier & Martel-Pelletier (1993); [b]Chèrie-Lignère et al (1990); [c]Bernareggi (1993); [d]Goodman & Gilman (1996); [e]Dromgoole et al (1982); [f]Hyneck et al (1988); [g]Vignon et al (1991); [h]Kurowski & Dunky (1988); [i]Verbeeck et al (1986); [j]Bayley et al (1987); [k]Ravis et al (1993); [l]Conroy et al (1991); [m]Noble & Balfour (1996); [n]Tuerck et al (1996); [o]Oberbauer et al (1993); [p]Glare & Walsh (1991); [q]Forrest et al (1982).

results in a more effective and persistent inhibition of MMP activity.

By using patients' synovial fluid, specimens of which were able to cleave our synthetic substrate, we found an inhibitory effect comparable with the IC50 values calculated for nimesulide and meloxicam. Although this finding seems to indicate that our in-vitro approach might be applied on synovial fluid from man, more detailed trials will be performed to transform our in-vitro approach to a direct check on synovial fluid. This is because the collagenase has a broad substrate specificity for ex-vivo checks, a more specific fluorescent peptide should probably be chosen and because the level of collagenase in rheumatoid arthritis patients is highly variable, i.e. from 0–2·4 $\mu g\,mL^{-1}$ (Dieppe et al 1988).

Previous observations demonstrated that some NSAIDs might suppress collagenase (Vignon et al 1990, 1991); even if the mechanism proposed was correlated with MMP synthesis (Pelletier & Martel-Pelletier 1993) the in-vitro inhibition of mature collagenase reported in this study might be concomitant with or an alternative to those studies. In conclusion, our data indicate that some NSAIDs act as inhibitors of MMP and might consequently help reduce pathological cartilage catabolism. Nevertheless, their possible pharmacological role as collagenase inhibitors in such collagen degradative diseases remains to be established by clinical studies.

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