Isothiazolones interfere with normal matrix metalloproteinase activation and inhibit cartilage proteoglycan degradation

Elizabeth C. ARNER*, Michael A. PRATTA, Bruce FREIMARK, Michael LISCHWE, James M. TRZASKOS, Ronald L. MAGOLDA and Stephen W. WRIGHT

Inflammatory Diseases Research, The DuPont Merck Pharmaceutical Company, Experimental Station, Wilmington, DE 19880-0400, U.S.A.

A series of isothiazolones that inhibit pro-(matrix metalloproteinase) (proMMP) activation but do not inhibit the active enzyme are effective as cartilage protectants in bovine nasal cartilage organ culture, preventing interleukin-1 (IL-1)-induced proteoglycan (aggrecan) degradation without affecting its synthesis. These compounds were found to bind to prostromelysin (proMMP-3) in a non-dialysable and stoichiometric manner. Preincubation with cartilage-protectant isothiazolones prevented the binding of [¹⁴C]iodoacetamide to Cys⁷⁵ of the MMP-3 propeptide, suggesting that the activity of these compounds involves their binding to the Cys⁷⁵ of the MMP zymogen. Studies following chymotrypsin activation of proMMP-3 by SDS/PAGE indicated that altered processing of the 57 kDa zymogen to the

active form occurred in the presence of compound. The 53 kDa intermediate seen on normal activation was not formed; instead a different intermediate appeared with a molecular mass of approx. 46 kDa. N-terminal sequence analysis indicated that this intermediate was formed by cleavage at the putative 4-aminophenylmercuric acid cleavage site. Importantly the 45 kDa active MMP-3 species formed in the presence of compound was one amino acid residue shorter than the native MMP-3. These results suggest that the inhibition of cartilage proteoglycan degradation by isothiazolones might be due to their ability to bind to the Cys⁷⁵ in the propeptide region of the MMP zymogen and interfere with its normal activation process.

INTRODUCTION

Arthritis is a disease of joints, characterized by gradual loss of the extracellular matrix of articular cartilage. One of the earliest changes observed in arthritis is depletion of the high-molecular-mass aggregating proteoglycan (aggrecan) from cartilage [1]. This loss occurs despite an increase in the rate of synthesis, suggesting that there is an increased rate of proteoglycan breakdown. It is relevant to this suggestion that in osteoarthritic cartilage, aggrecan is altered in its biochemical properties as characterized by increased extractability, loss of ability to aggregate with hyaluronic acid and decrease in size [2,3]. These changes can be attributed to proteolytic digestion of the hyaluronic acid-binding region of the aggrecan core protein. Matrix metalloproteinases (MMPs), which are synthesized and secreted from connective tissue cells, have been shown to clip aggrecan in this region [4,5]. MMPs can degrade collagen and proteoglycan at physiological pH [6,7] and are thought to play a key role in the breakdown of the cartilage matrix.

Stromelysin (MMP-3), one member of this MMP family, has been shown to degrade a wide range of extracellular matrix components, including cartilage proteoglycan, fibronectin, type IV collagen, laminin and type IX collagen [4,5,8,9]. Elevated levels of MMP-3 and interstitial collagenase (MMP-1) have been found in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis [10,11]. In addition, MMPs that degrade proteoglycan have been isolated from human articular cartilage [12], and one of these enzymes was demonstrated to be immunologically identical with MMP-3 [13]. MMP-3 [14,15] and MMP-1 [16,17] levels have been shown to be elevated in human osteoarthritic cartilage where MMP activity in the cartilage correlated with overall disease severity and with cartilage site

relative to the lesion. In addition, immunohistochemical studies have demonstrated the presence of MMP-3 in connective tissue from both human rheumatoid arthritis and osteoarthritis joints [18,19].

All of the members of the matrix metalloproteinase family are produced as inactive zymogens that must undergo cleavage to the lower-molecular-mass active enzyme to be capable of degrading their respective substrates [20]. These proteases are thought to be held in the inactive form through coordination of a cysteine in the pro-region of the molecule with the active-site zinc ion, thereby blocking access to the active site and maintaining the enzymes in the inactive zymogen form [21,22]. Activation involves a conformational change in the proenzyme followed by the removal of the propeptide by a series of autocatalytic cleavages. Thus the activation of these proteases provides a potential control point for regulating their activity in breaking down the cartilage matrix.

Cartilage degradation in arthritis is thought to be mediated, at least in part, by interleukin 1 (IL-1), a cytokine that stimulates metalloproteinase synthesis by connective tissue [23,24]. Both *in vitro* [25–28] and *in vivo* [29–33], IL-1 causes a net decrease in the proteoglycan content of cartilage by both increasing the breakdown of proteoglycan and decreasing its synthesis. The effect on proteoglycan degradation has been attributed at least in part to IL-1's ability to up-regulate MMP synthesis in articular chondrocytes [23,34]. Therefore IL-1-stimulated proteoglycan degradation in cartilage in organ culture provides a model for evaluating compounds that act to block at any of a number of steps to prevent cartilage breakdown, including inhibition of the synthesis, activation or activity of MMPs.

Considerable efforts have been made to identify active-site inhibitors of MMPs. Indeed, a number of agents, mostly peptidic

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; IL-1, interleukin 1; MMP, matrix metalloproteinase; RA, rheumatoid arthritis.

* To whom correspondence should be addressed.

in nature, have been identified as active-site inhibitors of these proteases [35] and several of these compounds have been shown to be effective inhibitors of IL-1-induced cartilage degradation [36,37]. We have recently identified a series of N-aryl pyridofused isothiazolones that are simple, non-peptidic small molecule structures that inhibit IL-1-induced cartilage proteoglycan degradation [38]. These compounds are as potent, or more so, in this system as other anti-inflammatory agents [28] including MMP active-site inhibitors. However, they are not effective as inhibitors of MMP activity.

Here we demonstrate that this unique series of isothiazolones blocks normal activation of proMMP-3 and inhibits IL-1-induced cartilage degradation without affecting cartilage matrix synthesis. Further we provide results to suggest that this activity is due to their ability to bind to Cys⁷⁵ in the pro-region of the MMP zymogen and interfere with the normal activation of these proteases.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum, penicillin/streptomycin/amphotericin B, and neomycin were from Gibco (Grand Island, NY, U.S.A.). [³⁵S]Sulphate was from New England Nuclear (Boston, MA, U.S.A.). Chymotrypsin was from Worthington (Freehold, NJ, U.S.A.), PACK II was from Calbiochem (La Jolla, CA, U.S.A.) and iodoacetamide and PMSF were from Sigma (St. Louis, MO, U.S.A.). The IL-1 used was a soluble, fully active recombinant human IL-1 β produced as described previously [39]. The specific activity was 10⁷ units/mg of protein, with 1 unit being defined as the amount of IL-1 that generated half-maximal activity in the thymocyte proliferation assay. Purified proMMP-3 from human rheumatoid synovial fibroblasts [40] was a gift from Dr. Hideaki Nagase (University of Kansas Medical School, Kansas City, KS, U.S.A.). The C-terminal truncated form of human fibroblast proMMP-3 containing the propeptide domain and catalytic domain was expressed in *Escherichia coli* and purified to homogeneity. This protein consists of residues 1–255 of full-length proMMP-3 and when activated by (4-aminophenyl)mercuric acetate has catalytic activity equivalent to the native protein. The inhibitors XG076 (7-aza-2-phenylbenzisothiazol-3-one), XE551 (7-aza-2-phenylbenzisoselenazol-3-one) and XH524 [2-(*t*-butylthio)-*N*-phenylnicotinamide] were synthesized at DuPont Merck as previously described [38]. ¹⁴C-labelled XG076 was prepared in two steps from 2-benzylsulphanylnicotinic acid and [U-¹⁴C]aniline hydrochloride as described for the synthesis of unlabelled XG076 [38].

Tissue preparation

Nasal septa were removed from bovine noses obtained fresh at the time of slaughter. Uniform cartilage discs (1 mm thick, 8 mm in diameter) were prepared as described by Steinberg et al. [41] and cut into eighths.

Organ culture

Cartilage pieces were cultured as described previously [42]. Briefly, each eighth of a cartilage disc was weighed then placed into a well of a 96-well culture dish containing 180 μ l of DMEM supplemented with 5% (v/v) heat-inactivated fetal calf serum, 100 i.u./ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B and 50 μ g/ml neomycin. Paired explants from the same disc were used to compare the effects of various experimental conditions. Eight replicates per treatment group

were run for each experiment, and a well containing medium but no cartilage was included as a blank for each group. Cultures were incubated for 40 h at 37 °C in an atmosphere of air/CO₂ (19:1).

Cartilage degradation inhibitor studies

Compounds were dissolved at 10 mM in DMSO and further diluted with DMEM, supplemented as above, to the required concentrations. DMSO concentrations in the culture media never exceeded 1%; this concentration of DMSO when included with IL-1 had no effect on the alterations in cartilage proteoglycan metabolism induced by IL-1 alone. Cartilage was incubated in the absence or presence of 500 ng/ml IL-1, with or without compound, for 40 h. Under these conditions 500 ng/ml IL-1 resulted in submaximal stimulation of proteoglycan breakdown [28], thus allowing the observation of either inhibition or augmentation of the effects of IL-1 by the added agent. When included, compounds were present throughout the culture period.

At the end of the incubation, the media were removed for glycosaminoglycan analysis and replaced with Ham's F-12 medium, containing 20 μ Ci/ml [³⁵S]sulphate. The samples were incubated for a labelling period of 2 h. The media were then removed and the cartilage was evaluated as described previously [42]. Briefly, it was digested overnight with papain, the proteoglycan in the digest precipitated with cetylpyridinium chloride, and the precipitates counted for ³⁵S on a Packard Matrix 96 counter. [³⁵S]Sulphate incorporation was determined as d.p.m. per mg wet weight of cartilage.

Glycosaminoglycan (GAG) in the culture medium was measured from the amount of polyanionic material reacting with 1,9-Dimethylmethylene Blue [43], using shark chondroitin sulphate as a standard. Results were reported as μ g of GAG per mg wet weight of cartilage.

Thiol reactivity

Compounds (200 mM) in tetrahydrofuran were treated with an equal volume of a freshly prepared solution of 200 mM *t*-butyl mercaptan in tetrahydrofuran in a sealed flask under nitrogen, in the presence or absence of 1.0 equivalent of anhydrous zinc chloride. The progress of the reaction was followed by TLC. After 24 h, the reaction mixture was evaporated to dryness and the residue examined by [¹H]-NMR to determine the amount of mixed disulphide generated as a measure of the extent of the reaction.

MMP-3 assay

MMP-3 activity was assessed with a [³H]transferrin substrate as previously described [40]. Briefly, 10 μ l of [³H]transferrin substrate (1185 μ g/ml; specific radioactivity 14870 d.p.m./ μ g) was added to 40 μ l of sample to a final volume of 50 μ l in 0.1 M Tris/HCl, pH 7.8, containing 0.15 M NaCl, 10 mM CaCl₂ and 0.02% NaN₃, and incubated for 4 h at 37 °C. The reaction was quenched with 200 μ l of ice-cold 3.3% (w/v) trichloroacetic acid, the mixture centrifuged at 22 °C for 5 min in a Brinkman microcentrifuge at 16000 rev./min, and 100 μ l of the supernatant counted for radioactivity to measure trichloroacetic acid-soluble digestion products.

Activation studies

ProMMP-3 was activated with chymotrypsin in the presence or absence of compound. Activation was performed at 25 °C with an incubation time of 2 h, which in time-course studies resulted

in an increase in MMP-3 enzymic activity that was linear with respect to time and product formed. Concentrations of activator were based on the studies by Okada et al. [44]. Activation was initiated by adding 20 nM chymotrypsin to 110 nM proMMP-3 in 10 mM Tris/HCl, pH 7.5 containing 80 mM NaCl, 2 mM CaCl_2 , 0.01 % Brij35 and 0.004 % NaN_3 in a total volume of 16.6 ml. At the end of the activation period, chymotrypsin was quenched with a molar excess of PMSF (500 μM) and the amount of MMP-3 activity was evaluated with a [^3H]transferrin substrate. The concentration of PMSF used was shown to inhibit chymotrypsin activity completely.

Compounds, after being dissolved at 10 mM in DMSO and further diluted in water to the required concentrations, were added to proMMP-3 immediately before the activation step. The DMSO concentration never exceeded 1 % during activation and, at this concentration, DMSO had no effect on MMP-3 enzyme activity nor on the activity of the activators. To determine the direct effect of compounds on MMP-3 enzyme activity, proMMP-3 was activated in the absence of compound, and then compound was added after the activator was quenched but before evaluation of enzymic activity.

For dialysis studies, proMMP-3 was preincubated for 15 min with 100 μM XE551 or buffer alone. Samples were dialysed overnight against assay buffer and activated with chymotrypsin as described above.

HPLC analysis

Recombinant truncated human proMMP-3 (0.9 μM) was incubated with [^{14}C]XG076 (100 μM ; specific radioactivity 14 mCi/mmol) in 50 mM Tris, pH 8.0, containing 400 mM NaCl, 10 mM CaCl_2 , 0.05 % Brij-35 and 0.02 % NaN_3 for 1 h at 37 °C. An aliquot of the incubation mixture was applied to a Vydac C4 column (0.46 cm $\times$ 5.0 cm). The unbound [^{14}C]XG076 and the [^{14}C]XG076–proMMP-3 complex were eluted with a 0–65 % (v/v) gradient of acetonitrile in 25 min at a flow rate of 1 ml/min; absorbance at 220 nm was monitored. Fractions of 1 ml were collected and 100 μl aliquots of these samples were counted to determine the presence of ^{14}C , and the molar ratio of [^{14}C]XG076 to proMMP-3 protein in the complex was calculated.

[^{14}C]iodoacetamide-labelling studies

ProMMP-3 (1 μM) in TNC buffer (50 mM Tris, pH 8.0, containing 150 mM NaCl, 10 mM CaCl_2 , 0.05 % Brij-35 and 0.02 % NaN_3) alone or with 100 μM iodoacetamide, 100 μM XE551, 100 μM XG076 or 100 μM XH524 was incubated for 1 h at room temperature. Samples were then dialysed against three 1 litre volumes of TNC at 4 °C to remove unbound compound. Dialysed proMMP-3 samples were incubated for 15 min with 20 mM EDTA at room temperature, followed by labelling with 5 μCi of [^{14}C]iodoacetamide (Amersham; 57.6 $\mu\text{Ci}/\mu\text{mol}$) in 50 μl for 30 min at room temperature. Samples were subjected to electrophoresis on SDS/PAGE [10 % (w/v) gel] and stained with Coomassie Blue (R-250). The SDS/PAGE gel was soaked with autoradiographic intensifying emulsion (Autofluor; Amersham), dried and exposed to Kodak XAR film at –70 °C. Coomassie Blue staining and [^{14}C]iodoacetamide labelling of proMMP-3 was quantified by scanning densitometry. The percentage incorporation of [^{14}C]iodoacetamide into compound-treated proMMP-3 protein was calculated by the ratio of [^{14}C]iodoacetamide to Coomassie Blue densitometry values relative to the control proMMP-3 ratio, which was designated as 100 % [^{14}C]iodoacetamide incorporation.

Evaluation of proMMP-3 activation products

ProMMP-3 was activated with chymotrypsin in the presence of 100 mM XG076 under the conditions described above. Chymotrypsin was quenched with PMSF, and autocatalysis of proMMP-3 intermediates was inhibited by adding a large molar excess of EDTA (100 mM). The samples were dried, resuspended in SDS/PAGE loading buffer (Novex, San Diego, CA, U.S.A.) containing 5 % (v/v) 2-mercaptoethanol, and the proteins separated by electrophoresis on 10 % polyacrylamide gels (Novex, San Diego, CA, U.S.A.). Gels were stained by using a silver stain kit (Integrated Separation Systems, Natick, MA, U.S.A.) and band intensity was quantified by scanning densitometry.

N-terminal sequence analysis

The N-terminal sequence analysis by automated Edman degradation was performed on a Beckman PI2090 gas-phase sequencer with program 40 as recommended by the manufacturer (Beckman Instruments, Fullerton, CA, U.S.A.). The phenylthiohydantoin–amino acids were analysed with a Hewlett-Packard 1090L reverse-phase HPLC.

Statistical analysis

Significant differences between groups were tested by Duncan's multiple-range test when analysis of variance was significant ($P < 0.05$). Values are shown as means $\pm$ S.E.M.

RESULTS

Effect on IL-1-induced alterations in proteoglycan metabolism

From previous studies we found that IL-1 caused a time- and concentration-dependent stimulation of proteoglycan breakdown and inhibition of proteoglycan synthesis in cartilage organ culture [28]. To evaluate inhibitors, cartilage was stimulated with 500 ng/ml IL-1 for 40 h, which resulted in a submaximal effect on proteoglycan metabolism. A series of isothiazolones and isoselenazolones were found to inhibit IL-1-induced proteoglycan breakdown at micromolar concentrations [38]. XG076, which exemplifies this series, caused a concentration-dependent inhibition of proteoglycan degradation; the IC_{50} estimated from the concentration–response curve was 14 μM (Figure 1). IL-1

Figure 1 Inhibition of IL-1-induced cartilage proteoglycan release by XG076

Bovine nasal cartilage was incubated for 40 h in control medium or 500 ng/ml of IL-1 in the absence or presence of XG076. At the end of the culture period the amount of proteoglycan breakdown was determined by measuring the GAG released into the medium as μg of GAG released per mg wet weight of cartilage. The percentage inhibition of IL-1-stimulated GAG release is plotted versus inhibitor concentration. Abbreviation: μM , μM .

Table 1 Effect of XG076 on cartilage proteoglycan synthesis

Proteoglycan synthesis in cartilage from studies described in Figure 1 was determined after the 40 h incubation with the various treatments by labelling cartilage with [35 S]sulphate (2 h pulse) as described in the Materials and methods section. The drug concentration shown was that included during the 40 h cartilage culture in the presence of 500 ng/ml IL-1. Proteoglycan synthesis is expressed as d.p.m. of [35 S]sulphate incorporated per mg wet weight of cartilage. Values are means $\pm$ S.E.M. ($n = 8$).

Treatment	Drug concentration (μ M)	Proteoglycan synthesis (d.p.m./mg)
Control	0	1165 $\pm$ 121
IL-1	0	408 $\pm$ 65
IL-1 + XG076	3	417 $\pm$ 53
IL-1 + XG076	10	497 $\pm$ 69
IL-1 + XG076	30	515 $\pm$ 40
IL-1 + XG076	100	403 $\pm$ 44

Table 2 Thiol reactivity of isothiazolones and isoselenazolones

Compound	X	A	R ₁	Cartilage IC ₅₀ (uM)	Reactivity	
					C ₄ H ₉ SH alone	C ₄ H ₉ SH + ZnCl ₂
XG076	S	N	C ₆ H ₅	14	100% (15 min)	100% (15 min)
XE551	Se	N	C ₆ H ₅	5	100% (15 min)	100% (15 min)
XE117	Se	COCH ₃	C ₆ H ₅	3	10% (24 h)	100% (60 min)
XD682	S	CH	C ₆ H ₅	3	25% (24 h)	100% (15 min)
XH294	S	CCH ₃	C ₆ H ₅	>30	<5% (24 h)	10% (24 h)

decreased proteoglycan synthesis to approx. one-third of control levels and XG076 did not affect this rate of synthesis at concentrations up to 100 μ M (Table 1), where it totally blocked IL-1-induced PG breakdown. XG076 also caused no effect on proteoglycan synthesis in control cultures (results not shown).

Thiol reactivity

Pharmacokinetic studies on these compounds suggested that the principal route of metabolism occurred via reduction with excess glutathione, affording the ring-opened thiol (SH) or selenol (SeH) derivatives, which could undergo further metabolic transformation (S. W. Wright, unpublished work). Therefore the reactivity of a series of these compounds towards a model thiol was investigated. t-Butyl mercaptan was selected because its modest volatility (b.p. 65 °C) allows its ready removal from the reaction mixture, and its simple [1 H]-NMR spectrum allows accurate ($\pm 5\%$) estimation of the extent of disulphide formation. It was found that those compounds that were unstable in plasma underwent fast reaction with t-butyl mercaptan to yield the mixed disulphide; compounds that were stable in plasma underwent little or no reaction with t-butyl mercaptan. Results with selected compounds are shown in Table 2. Compounds that underwent appreciable reaction (more than 50%) with t-butyl mercaptan within 24 h were also active in blocking proteoglycan degradation as illustrated by XG076 and XE551. This suggested these compounds might exert their effect via a nucleophilic attack at the isothiazolone sulphur atom. Certain exceptions existed, such as XE117 and XD682, which were unreactive

Table 3 Effect of cartilage protectants on proMMP-3 activation

ProMMP-3 was activated with 20 nM chymotrypsin for 2 h at 25 °C in the absence (control) or presence of 100 μ M compound. At the end of the incubation, chymotrypsin activity was quenched with PMSF, and the amount of active MMP-3 generated was evaluated with a [3 H]transferrin substrate. Values are means $\pm$ S.E.M. * Significantly different from control at $P < 0.05$

Treatment	10 ⁻³ $\times$ MMP-3 activity (d.p.m.)
Control	36.4 $\pm$ 4.8
XE551	17.2 $\pm$ 1.2*
XH524	42.8 $\pm$ 2.5
XE551 (after activation)	40.6 $\pm$ 1.2

towards t-butyl mercaptan but still blocked proteoglycan breakdown. However, when the reactions with t-butyl mercaptan were repeated in the presence of zinc ion as a catalyst, it was found that the previous exceptional compounds underwent reaction with t-butyl mercaptan, whereas compounds that were inactive in blocking proteoglycan degradation, such as XH294, still failed to react with the mercaptan to an appreciable extent.

Effect on proMMP-3 activation

On the basis of the thiol reactivity, the effects of zinc ion on the thiol reactivity, and the structure–activity relationships of these compounds [38], we hypothesized that their mechanism of action involved binding to Cys⁷⁵ in proMMP-3 and interfering with its conversion to the mature, active enzyme. To test this hypothesis we activated proMMP-3 with chymotrypsin in the presence or absence of compound. At the end of activation, PMSF was added to quench the chymotrypsin activity and the amount of active MMP-3 generated was evaluated with [3 H]transferrin as a substrate. Because the compounds were also present during the enzyme assay, an effect by the compound on the activation of proMMP-3 would not be distinguished from an inhibition of the active enzyme. Therefore, to separate these activities, proMMP-3 was activated in the absence of compound, and then compound was added before enzymic evaluation in the transferrin assay to assess the direct effect of compounds on the active enzyme.

As illustrated by XE551, cartilage-protectant isothiazolones interfered with the generation of MMP-3 activity by chymotrypsin (Table 3). In contrast, XH524, a structurally related compound lacking cartilage-protectant activity, did not affect activation. The lack of effect on MMP-3 activity when XE551 was added after activation (i.e. present only during the enzymic assay) demonstrates that these compounds do not inhibit the action of the active enzyme on its substrate. To ensure that the compounds were not acting by inhibiting the chymotrypsin used to activate proMMP-3, we tested their effect on chymotrypsin activity in the transferrin assay. At concentrations up to 100 μ M the compounds had no effect on chymotrypsin (i.e. control, 9100 $\pm$ 235 d.p.m.; 100 μ M XE551, 10960 $\pm$ 2430 d.p.m.). We also evaluated the inhibitory effect of compounds by using plasmin as the activator and found a similar decrease in the amount of MMP-3 activity generated (results not shown). Thus the ability of these agents to interfere with normal MMP activation was independent of the activator used.

Evidence for isothiazolone binding to Cys⁷⁵ of proMMP-3

When proMMP-3 was preincubated with buffer alone or the cartilage protectant XE551 (100 μ M), then dialysed overnight to remove unbound drug, the inhibition of activation persisted

Figure 2 HPLC evaluation of incorporation of [^{14}C]XG076 into proMMP-3

Recombinant truncated proMMP-3 was incubated in the presence of 100 μM [^{14}C]XG076; the reaction mixture was separated by HPLC and assessed by absorbance at 220 nm and fractions were counted for radioactivity. The peak migrating at 13.5 min is [^{14}C]XG076 and that at 25.7 min is a complex of [^{14}C]XG076 and proMMP-3.

(control, 1510 ± 489 d.p.m.; XE551, 469 ± 21 d.p.m.), suggesting that the isothiazolones bind covalently to proMMP-3.

We also used a radiolabelled isothiazolone to demonstrate incorporation of these compounds into proMMP-3. For these studies recombinant truncated proMMP-3 was incubated in the presence of [^{14}C]XG076, the reaction mixture was separated by HPLC and fractions were collected and counted for radioactivity. In Figure 2 the absorbance at 220 nm and the radioactive profile are illustrated. The peak migrating at 13.5 min is [^{14}C]XG076 and the peak migrating at 25.7 min is a complex of [^{14}C]XG076 and proMMP-3. On the basis of the specific radioactivity of [^{14}C]XG076 (14 mCi/mmol), the amount of radioactivity (4700 c.p.m.) incorporated into the proMMP-3 corresponds to a 1:1 molar ratio of drug (0.151 nmol) to proMMP-3 protein loaded (0.163 nmol).

To test the hypothesis that inhibition of normal proMMP-3 activation involves the binding of these compounds to Cys⁷⁵ in the pro-region of the zymogen, we employed the thiol acylating agent iodoacetamide. This compound binds specifically to Cys⁷⁵ of proMMP-3 in the presence of EDTA [45]. Thus if isothiazolones act by binding to Cys⁷⁵, their binding should prevent binding of iodoacetamide to this residue. ProMMP-3 was pre-incubated with non-radiolabelled iodoacetamide or isothiazolones and then labelled with [^{14}C]iodoacetamide in the presence of 20 mM EDTA and evaluated by SDS/PAGE analysis. Figure 3 shows the proMMP-3 protein bands stained with Coomassie Blue and the autoradiography illustrating ^{14}C incorporation into proMMP-3. Band intensity was determined by scanning densitometry of these blots and the ratio of ^{14}C to total protein calculated relative to that of the control unmodified proMMP-3 ratio (control, 1.0; iodoacetamide, 0.03; XG076, 0.22; XH524, 0.95). As expected, preincubation with iodoacetamide prevented subsequent labelling with [^{14}C]iodoacetamide. XG076, which blocks both proteoglycan degradation and proMMP-3 activation, decreased [^{14}C]iodoacetamide incorporation, indicating that it is bound to Cys⁷⁵. In contrast, XH524, a structurally related but inactive compound, did not. These results suggest that inhibition of

Figure 3 Evaluation of the ability of cartilage protectants to prevent binding of [^{14}C]iodoacetamide to proMMP-3

ProMMP-3 was preincubated with or without 100 μM unlabelled iodoacetamide or isothiazolone, dialysed, labelled with [^{14}C]iodoacetamide in the presence of 20 mM EDTA and analysed by SDS/PAGE. Protein bands were stained with Coomassie Blue and ^{14}C incorporation was evaluated by autoradiography.

proMMP-3 activation by these compounds involves binding to Cys⁷⁵.

Analysis of proMMP-3 processing

SDS/PAGE analysis after the activation of proMMP-3 showed that when drug was present during activation with chymotrypsin, the normal 53 kDa MMP-3 intermediate was not formed (Figure 4). Instead a different intermediate appeared with a molecular mass of approx. 46 kDa. To determine the effect of compound on the rate of conversion of proMMP-3, the time course of activation was followed by SDS/PAGE analysis (Figure 5A). Analysis of blots from time-course studies by scanning densitometry indicated that the rate of disappearance of the 57 kDa proMMP-3 was not altered, nor was the rate of appearance of the mature, active form of the enzyme that was represented by the band at 45 kDa (Figure 5B).

N-terminal sequence of proMMP-3 activation products

The N-terminal sequence of the 46 kDa MMP-3 intermediate, formed only in the presence of drug, was VMRKP. This sequence indicates that cleavage by chymotrypsin occurred at the putative first cleavage site for 4-aminophenylmercuric acid activation,

Figure 4 Effect of isothiazolones on proMMP-3 processing

ProMMP-3 was activated with chymotrypsin as described in Table 3, in the absence or presence of 100 μM XG076. After 2 h the reaction was quenched with PMSF, autocatalysis of proMMP-3 intermediates was blocked with EDTA, and proteins were analysed by SDS/PAGE with silver staining. Lane 1, proMMP-3 before activation; lane 2, proMMP-3 activated in the absence of XG076; lane 3, proMMP-3 activated in the presence of XG076. Abbreviation: kD, kDa.

Figure 5 Time course of effect of cartilage protectants on proMMP-3 processing

ProMMP-3 was activated with chymotrypsin as described in Table 3 in the absence or presence of 100 μ M XG076. (A) At various times the reaction was quenched with PMSF and proteins were analysed by SDS/PAGE with silver staining. Abbreviation: kD, kDa. (B) The band intensities of the 57 kDa MMP-3 zymogen (●), the 53 kDa intermediate (▲) or 46 kDa intermediate (▼) and the 45 kDa mature MMP-3 (■) were quantified by scanning densitometry and band intensity plotted against activation time.

Figure 6 Cleavage sites of proMMP-3 in the absence and presence of inhibitor

N-terminal sequence analysis was performed on the 46 kDa MMP-3 intermediate and 45 kDa mature MMP-3 formed in the presence of inhibitor. Cleavage sites are indicated by arrows. Abbreviations: kD, kDa; chymo, chymotrypsin; APMA, 4-aminophenylmercuric acid.

located at the Glu⁶⁸-Val⁶⁹ bond (Figure 6). This differs from where chymotrypsin normally clips proMMP-3 in the 'bait region' between Phe³⁴ and Val³⁵. Importantly, the N-terminal sequence of the 45 kDa MMP-3 formed in the presence of XE551 was RTFPG, rather than FRTFPG as seen in the absence of drug. This suggests that proMMP-3 was clipped between Phe⁸³ and Arg⁸⁴ in the presence of drug rather than at the His⁸²-Phe⁸³ bond, resulting in a protein one residue shorter than the native MMP-3.

DISCUSSION

We have demonstrated that a series of isothiazolones and isoselenazolones are active in blocking IL-1-induced proteoglycan breakdown without inhibiting proteoglycan synthesis in a cartilage organ culture system. The activity of these compounds is unique in comparison with other anti-arthritic drugs in that non-steroidal anti-inflammatory drugs have generally been shown to be inactive in blocking proteoglycan breakdown in this system, and other 'disease-modifying' compounds are either inactive or block proteoglycan breakdown only at concentrations where they also cause additional inhibition of proteoglycan synthesis [28,46,47]. For example, indomethacin, naproxen, ibuprofen and phenylbutazone were all inactive at 30 μ M in this system; tetracycline was also inactive at 30 μ M; whereas the British Biotech MMP active-site inhibitor, BBT-16, was active with an IC₅₀ of 5 μ M (E. C. Arner, unpublished work). The profile of activity of the isothiazolones, i.e. inhibition of proteoglycan breakdown without affecting synthesis, suggests that they are not directly inhibiting IL-1. We have found that they do not inhibit IL-1 receptor binding (E. C. Arner, unpublished work) and are therefore not working as receptor antagonists of IL-1. Instead they appear to be affecting only the IL-1-stimulated proteoglycan breakdown, which is consistent with a mechanism of action based on inhibition of MMP.

Results on reactivity of the isothiazolones suggested that these compounds act via reaction at the isothiazolone sulphur atom, possibly through a nucleophilic attack at sulphur. On the basis of the reactivity relationships observed we hypothesized that these compounds may act by blocking the normal activation of the MMP zymogen to the mature, active enzyme. It is proposed that these compounds interact with the 'cysteine switch' that is present in the MMP zymogen [22]. Normally, proMMPs, zinc-containing proteinases, are secreted with a peptide chain containing a free cysteine residue in which the cysteine thiol group is co-ordinated to the zinc (Figure 7A). This effectively blocks the active site and keeps the proMMP in the inactive state. During proteolytic activation the propeptide is cleaved at the 'bait region' by an activating protease, and the cysteine thiol dissociates from the zinc. This process exposes the active site, allowing bimolecular autocatalysis to occur to form the mature active MMP. We propose that the isothiazolones react with the cysteine thiol residue, forming a disulphide bond (Figure 7B). This reaction can be catalysed by the enzyme's zinc ion, resulting in arylcarboxanilide formation. The adduct may then act as a ligand for zinc, replacing the cysteine thiol group in this role. In turn, this reaction could interfere with the normal activation of the proMMP to the active enzyme.

To test this hypothesis we examined the effect of the isothiazolones on proMMP-3 activation. Our results show that these compounds interfere with normal activation of proMMP-3 to the mature enzyme and that they do not inhibit the activated MMP-3. When proMMP-3 is activated in the presence of these cartilage-protectant compounds, they inhibit the generation of MMP-3 activity. However, if they are added after activation, and

Figure 7 Mechanism of inhibition of proMMP-3 activation by isothiazolones

(A) In the absence of inhibitor, activation of the propeptide involves the dissociation of the cysteine thiol from the zinc ion, which exposes the active site allowing bimolecular autocatalysis to occur to form the active mature MMP-3. (B) Isothiazolones react with the cysteine thiol residue, forming a disulphide bond. This adduct can then act as a ligand for zinc, replacing the cysteine thiol group and in turn interfering with the normal activation of proMMP-3 to the active enzyme. Abbreviations: kD, kDa; APMA, 4-aminophenylmercuric acid.

therefore are present only during the enzymic assay, they do not inhibit the activity of the mature MMP-3. We also found these compounds as a class to be inactive as inhibitors of cyclooxygenase (IC_{50} generally more than $750 \mu M$), phospholipase A2 (IC_{50} generally more than $1 mM$) and 5-lipoxygenase (IC_{50} generally more than $25 \mu M$) (E. C. Arner, W. Galbraith and R. Harris, unpublished work).

The compounds do not prevent the generation of MMP-3 activity by blocking the activating enzyme, but rather seem to alter the proMMP-3 substrate to prevent normal activation or processing. Evidence for this is the generation of a different MMP-3 intermediate of 46 kDa formed by cleavage at Glu⁶⁸-Val⁶⁹ during chymotrypsin activation of the isothiazolone-derivatized proenzyme rather than the 53 kDa intermediate formed on activation by chymotrypsin in the absence of compound. A similar 46 kDa intermediate is seen when plasmin is used to activate the isothiazolone-derivatized proMMP-3 (results not shown), suggesting that this alteration in cleavage site is not unique to chymotrypsin activation. However, whether chymotrypsin or MMP-3 is responsible for the cleavage of the isothiazolone-derivatized proenzyme at Glu⁶⁸-Val⁶⁹ is unclear. Although no change in the MMP-3 protein banding pattern is seen after incubation of the derivatized proenzyme for 16 h in the absence of chymotrypsin, chymotrypsin does not normally recognize a Glu residue at the P1 site. Therefore because a slight, transient increase in the 53 kDa intermediate is seen on activation of the derivatized proMMP-3 (Figure 5A), this intermediate might be formed and rapidly converted to 46 kDa by a bimolecular reaction of MMP-3.

Processing of the isothiazolone-derivatized proMMP-3 to the mature 45 kDa form one residue shorter than that produced on normal activation results from cleavage at the Phe⁸³-Arg⁸⁴ bond, which is consistent with chymotrypsin specificity. This suggests that either an MMP-3 with reduced activity failed to act at the His⁸²-Phe⁸³ bond or that binding of the isothiazolones to proMMP-3 results in a conformational change allowing the Phe⁸³-Arg⁸⁴ bond to be more susceptible to the activating proteases chymotrypsin and plasmin, leading to the formation of a mature form of the enzyme that might be less active than native

MMP-3. It has been demonstrated that a form of MMP-3 with two residues deleted from the N-terminus displays greatly diminished activity compared with the native enzyme (H. Nagase, personal communication). It is interesting to speculate that this might also be the case with MMP-3 having a single residue deleted from the N-terminus, which would explain the decrease in enzymic activity observed when activation takes place in the presence of these isothiazolone inhibitors. Studies to examine the activity of the 45 kDa form of MMP-3 formed in the presence of inhibitor are planned.

That this inhibition of activation involves binding of the isothiazolones or isoselenazolones to Cys⁷⁵ of proMMP-3 is supported by our results showing that when the cartilage-protectant compounds are preincubated with proMMP-3, they prevent binding of labelled iodoacetamide, suggesting that they are bound to Cys⁷⁵ and therefore prevent the interaction of [¹⁴C]iodoacetamide with this residue. Further evidence for the binding of these compounds to proMMP-3 is provided by results showing that a radiolabelled isothiazolone was incorporated into the zymogen in a stoichiometric manner. Interestingly, the isothiazolones apparently react with Cys⁷⁵ without EDTA, unlike the reaction with iodoacetamide where EDTA is required to facilitate binding. This might be due to the zinc ion's catalysing the interaction of the isothiazolone with the cysteine thiol residue to form an arylcarboxanilide adduct, which could then act as a ligand for the zinc, replacing the cysteine thiol in this function, whereas this would not occur on acetylation of the cysteine by iodoacetamide.

Results reported here support a mechanism of action for these cartilage-protectant compounds that is based on their inhibition of normal proMMP activation. Thus interfering with the activation of the matrix metalloproteinases may offer a control point for regulating their activity in breaking down the cartilage matrix. Although our studies examined the effect of isothiazolones on MMP activation by using proMMP-3 as the zymogen, the MMP family shares a common activation mechanism involving displacement of the cysteine-zinc interaction. Therefore it is expected that these compounds would act similarly to inhibit activation of the entire MMP family.

These isothiazolones/isoselenazolones provide a unique set of cartilage-protectant compounds that are capable of blocking cartilage proteoglycan degradation without decreasing proteoglycan synthesis; their potential as therapeutic agents for preventing the loss of cartilage in arthritis is being pursued.

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