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THE CHONDROPROTECTIVE DRUGS, ARTEPARON AND SODIUM PENTOSAN POLYSULPHATE, INCREASE COLLAGENASE ACTIVITY AND INHIBIT STROMELYSIN ACTIVITY *IN VITRO*

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Abstract—The effects of the chondroprotective drugs, sodium pentosan polysulphate (SP54) and Arteparon (glycosaminoglycan polysulphate), on the *in vitro* activities of the purified matrix metalloproteinases interstitial collagenase (matrix metalloproteinase 1, MMP1) and stromelysin (MMP3) were examined. Both drugs produced concentration-dependent enhancement of the degradation of type I collagen fibrils by purified human fibroblast collagenase and rat tumour collagenase. Rat collagenase activity was increased by drug concentrations above 0.5 μg/mL, whereas human collagenase activity was only increased by higher drug concentrations, above 5 μg/mL. The concentration dependence of the increase in rat collagenase activity was similar for both drugs, with a maximal 3-fold increase at 50 μg/mL. In contrast, human collagenase activity was increased to a greater extent by SP 54 compared to Arteparon, with maximal increases at 5000 μg/mL of 6-fold and 2–4-fold, respectively. Both drugs produced concentration-dependent inhibition of the proteoglycan-degrading activity of both human fibroblast stromelysin and rat tumour stromelysin. Rat and human stromelysin activities were inhibited at drug concentrations above 0.005 μg/mL, with a similar concentration dependence for both drugs. Fifty percent inhibition of rat stromelysin was produced by concentrations of each drug in the 0.5–5 μg/mL range. The pattern of inhibition of human stromelysin was similar, except that drug concentrations in the 500–5000 μg/mL range produced 50% inhibition. The possible modes of action for these drug effects and their possible pharmacological significance are discussed.

Arteparon (glycosaminoglycan polysulphate) and sodium pentosan polysulphate (SP 54),¶ Cartrophen, Anarthron) are sulphated polysaccharides comprised of repeating disaccharide units, similar to some naturally occurring polysaccharides. Arteparon is an over-sulphated chondroitin with an average molecular mass (M_r) of 10,000, while SP54 is a sulphated xylan fraction (average M_r 6000) comprised of a linear polypentose chain in which the majority of hydroxyl groups have been esterified with sulphate [1].

Arteparon and SP54 inhibit or slow the degradation of cartilage occurring in animal models of osteoarthritis, and also promote the repair of damaged cartilage [1–4]. Both drugs are also inhibitors of the degradative enzymes polymorphonuclear leukocyte elastase and cathepsin B; in addition, each drug inhibits a range of other hydrolases responsible for extracellular matrix degradation [1, 2]. Repair processes stimulated *in vitro* by these drugs include collagen, proteoglycan and hyaluronidase synthesis by synovial fibroblasts and chondrocytes [1, 2, 4]. Preliminary clinical trials of both drugs in osteoarthritis have indicated some beneficial effects [1], and further clinical trials of SP54 are currently being conducted in the investigating laboratory.

Degradation of collagen and, particularly, proteoglycan is a major feature of the pathology of osteoarthritis [5, 6]. This degradation is catalysed by a number of enzymes, including the matrix metalloproteinases interstitial collagenase (MMP1) and stromelysin (MMP3), which are thought to contribute significantly to the breakdown of collagen and proteoglycan, respectively [7].

It is not known whether sulphated polysaccharide chondroprotective drugs such as SP54 and Arteparon affect the activity of interstitial collagenase and stromelysin. Any effects of chondroprotective drugs on the activities of these enzymes could be of pharmacological significance.

In this report, we examine the effects of Arteparon and SP54 upon the *in vitro* activities of collagenase and stromelysin purified from both human and rat cell lines. We show for the first time that these chondroprotective drugs increase the degradation of type I collagen fibrils by collagenase, and inhibit the degradation of proteoglycan by stromelysin.

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¶ Abbreviations: SP54, sodium pentosan polysulphate; MMP, matrix metalloproteinase; SBTI, soy bean trypsin inhibitor.

MATERIALS AND METHODS

Materials. Drugs were obtained from the following sources: Arteparon from Luitpold-Werk, Munich, Germany; and SP54 from Arthropharm Pty Ltd, Sydney, Australia. TPCK-trypsin and soy bean trypsin inhibitor (SBTI) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents were of analytical grade.

Enzymes. Rat collagenase and stromelysin [8] were purified to electrophoretic homogeneity from the conditioned culture medium of the BC1 rat mammary carcinoma cell line as described previously [9]. Both enzyme activities were fully activated during purification as described [8]. The specific activities of these enzymes in the assays described below were 610 μg collagen degraded/min/mg enzyme (collagenase), and 160 μg proteoglycan released/min/mg enzyme (stromelysin).

Human procollagenase and prostromelysin were purified to electrophoretic homogeneity from skin fibroblast culture medium as described [10, 11]. Procollagenase (90 μg/mL) was activated by incubation with TPCK-trypsin (100 μg/mL) for 10 min at 25°, followed by incubation with SBTI (2 mg/mL) for 10 min at 25°. Prostromelysin (150 μg/mL) was activated by incubation with TPCK-trypsin (7.5 μg/mL) for 15 min at 37° followed by incubation with SBTI (75 μg/mL) for 5 min at 25°. This procedure results in mainly the 45 kDa active form of stromelysin. The specific activities of these enzymes in the assays described below were 280 μg collagen degraded/min/mg enzyme (collagenase), and 3.4 μg proteoglycan released/min/mg enzyme (stromelysin).

The collagenolytic and proteoglycan-degrading activities of the collagenases and stromelysins (both rat and human) were confirmed to be catalysed by metalloproteinases, since all activities were completely inhibited by addition of EDTA to a final concentration of 20 mM.

Enzyme assays. Collagenase activity was measured using fibrillar type I collagen substrate bound to microtiter wells [12]. In this assay, the amount of undegraded collagen remaining after assay incubation is quantitated by staining with Coomassie blue R-250 and measuring the absorbance of the resulting blue film at 590 nm. Where collagen has been degraded and/or solubilized, the absorbance is decreased compared to buffer-only controls.

Relative activities were quantitated by determining the concentration of collagenase which degraded 50% of the collagen. To achieve this, serial 2-fold dilutions of collagenase (control samples diluted in buffer only, or test samples diluted in buffer containing a particular concentration of drug) were assayed and the resulting absorbances were plotted against dilution factor. The dilution of sample which degraded 50% of the collagen was then determined by interpolation. Assays were terminated by washing with water and adding stain solution.

When the collagenase substrate was incubated with active trypsin (1 μg/well), up to 18% of the collagen was solubilized, confirming our earlier report that the collagen is predominantly native [12].

Stromelysin activity was measured using ^{3}H-labelled bovine proteoglycan trapped within polyacrylamide beads, essentially as described by Nagase and Woessner [13]. Assay incubations were performed in sealed 96-well plates containing 60 μL of enzyme and/or drug as appropriate, plus 40 μL of washed substrate slurry (7.5 mg beads/mL), all in assay buffer (see below). Assay reactions were terminated by placing plates on ice and adding 200 μL of ice-cold water per well. Plates were then shaken for 1 min, the contents centrifuged at 500 *g* for 5 min and 200 μL aliquots of supernatant taken from each well for scintillation counting of radioactivity. Relative enzyme activities were quantitated by reference to a standard curve. The substrate contained 150 μg proteoglycan/mg of total bead weight, with an activity of 280 dpm/μg proteoglycan.

All assays were incubated at 35° for 16 hr. The buffer used for both assays was 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl, 0.05% (w/v) Brij-35, 0.02% (w/v) NaN_3.

Statistical analysis. Enzyme activities measured in the presence of drugs (mean ± SD) were compared to control values using Student's *t*-test.

RESULTS

Collagenase (MMP1)

Both SP54 and Arteparon caused statistically significant, concentration-dependent increases in the activity of rat collagenase and human collagenase (Fig. 1). From the the results in Fig. 1, it was apparent that: (a) rat collagenase activity was increased by SP54 and Arteparon concentrations above 0.5 μg/mL, whereas human collagenase activity was increased by higher concentrations of both drugs (above 5 μg/mL). (b) Both drugs produced similar maximal increases in rat collagenase activity (2–3-fold), whereas SP54 produced a greater maximal increase (5–6-fold) than Arteparon (2–4-fold) in human collagenase activity. (c) Human collagenase activity continued to increase with higher drug concentrations, whereas rat collagenase activity was not increased further by drug concentrations above 50 μg/mL.

In the absence of collagenase, incubation of both drugs at the higher concentrations with collagen for 16 hr produced slight decreases in the final absorbance of the collagen substrate. Absorbances were decreased by 10% and 25%, compared to buffer-only controls, by drug concentrations of 500 and 5000 μg/mL, respectively. The drug concentrations producing this effect were 100–1000 times higher than those required to produce the same absorbance decreases in the presence of collagenase.

Stromelysin (MMP3)

Whereas both drugs increased collagenase activity, they produced statistically significant, concentration-dependent inhibition of stromelysin (Fig. 2). From these results it was apparent that: (a) the concentration dependence of inhibition of rat stromelysin showed a decrease in activity at drug concentrations of 0.05–0.5 μg/mL, a stabilization or slight increase in activity at 5–50 μg/mL and a further decrease in activity at drug concentrations of 500–

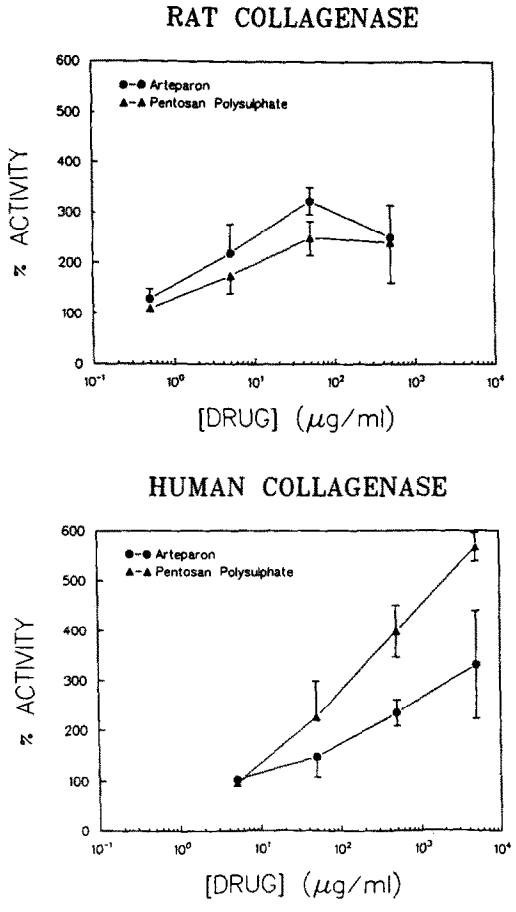

Fig. 1. Effects of SP54 and Arteparon on rat and human collagenase activities. Results are shown as means ± SD (N = 6). All results shown are significantly different ($P < 0.05$) from control (100%) activity.

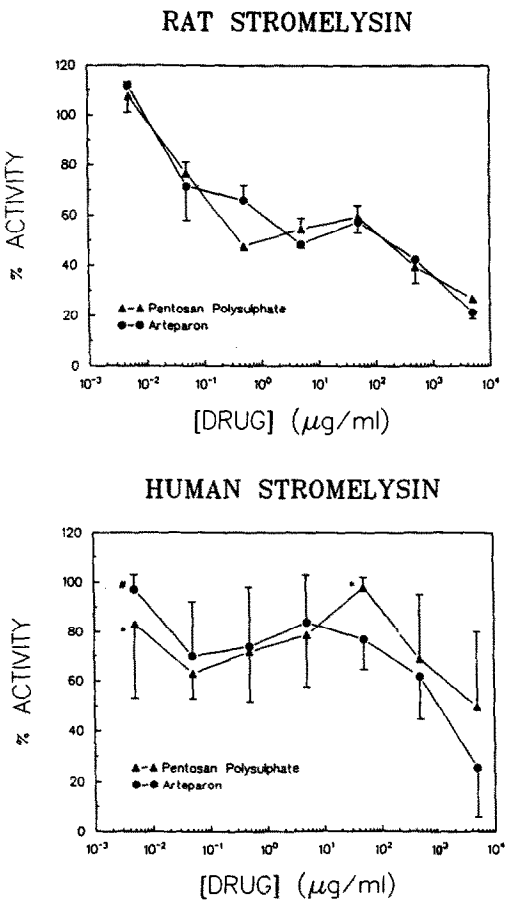

Fig. 2. Effects of SP54 and Arteparon on rat and human stromelysin activity. Results are shown as means ± SD (N = 6). Unless otherwise indicated, all results shown are significantly different ($P < 0.05$) from control (100%) activity.

5000 μg/mL. (b) The concentration dependence of inhibition of human stomelysin was similar to that of rat stromelysin, showing a decrease in activity at a drug concentration of 0.05 μg/mL, an increase towards control levels at 0.5–50 μg/mL and a further decrease in activity at drug concentrations of 500–5000 μg/mL. (c) Fifty percent inhibition of activity occurred at drug concentrations of 0.5–5 μg/mL for rat stromelysin and 500–5000 μg/mL for human stromelysin. (d) Standard deviations were greater for human stromelysin, probably due to the very low activity of this enzyme against the entrapped [^{3}H]proteoglycan substrate.

In the absence of stromelysin, the drugs caused no change in the release of radiolabelled proteoglycan from the substrate beads at all drug concentrations shown in Fig. 2.

DISCUSSION

The effects of SP54 and Arteparon on the activities of collagenase and stromelysin suggest that these drugs interact with the MMPs and/or their substrates. Evidence in the literature supports this suggestion. SP54 binds to a number of extracellular matrix components including type I collagen and, less strongly, proteoglycan [14]. Heparin, a sulfated polysaccharide like SP54 and Arteparon, binds to a range of collagen types [15–18] including type I and to collagenases [8, 19]. Both collagenase and stromelysin contain a putative heparin binding site [20]. We have observed that heparin and other highly sulphated polysaccharides, but not their unsulphated analogues, have similar effects to SP45 and Arteparon on collagenase and stromelysin activities (unpublished observations).

Collagenase (MMP1)

The results presented in this report suggest that SP54 and Arteparon interact with both collagenase and its fibrillar collagen substrate.

Figure 1 illustrates that the drugs had quantitatively different effects on rat collagenase activity compared

to human collagenase activity. The drug concentrations required to increase rat collagenase activity were lower than those required to increase human collagenase activity. Also, SP54 produced greater increases in human than rat collagenase activity. These findings suggest that the drugs interact with both collagenases, but with different affinities and effects, and perhaps at different sites.

In the absence of collagenase, both drugs also had effects on the fibrillar collagen substrate, producing decreases in the absorbance of the collagen after staining with Coomassie blue R-250. The drug concentrations producing this effect were much higher than the lowest drug concentrations required to increase collagen degradation by collagenase. Hence, it appears that the effect of the drugs on collagen degradation by collagenase is not solely due to the observed effect of the drugs on collagen.

However, it has been reported that highly sulphated polysaccharides, at concentrations which we have shown will increase collagenase activity, can alter the stability and microscopic morphology of type I collagen fibril networks [17, 21]. Both of these reports show that the addition of heparin (but not polysaccharides which are less highly sulphated) to type I collagen fibrils produces a more open network of distinct, larger diameter collagen fibrils than in controls. Perhaps the collagen in this open network is more readily accessible to and/or degraded by collagenase.

Alternatively, highly sulphated polysaccharides may facilitate the interaction between collagenase and collagen by acting as a template or by inducing a conformational change in collagenase which enhances its ability to degrade collagen. These two models are analagous to those proposed to explain the enhanced binding of thrombin to antithrombin caused by heparin [22].

Stromelysin (MMP3)

The inhibition of stromelysin by both drugs may arise through interactions between the drugs and stromelysin, and/or between the drugs and the proteoglycan substrate. The results presented here do not support one possibility over the other. However, by analogy with published data on the binding of SP54 to proteoglycan and elastase, we suggest that drug–enzyme interaction is more likely. Andrews *et al.* [14] reported that 50% inhibition of human polymorphonuclear leukocyte elastase was produced by SP54 concentrations in the same range as those giving 50% inhibition of rat stromelysin in Fig. 2. In the same report, these authors showed that the binding of SP54 to proteoglycan was too weak to account for elastase inhibition. Therefore, the binding of SP54 to proteoglycan is probably not strong enough to account for the inhibition of rat stromelysin by SP54.

The plateau in the inhibition curve for rat stromelysin, at drug concentrations of 5–50 μg/mL, may indicate the presence of more than one inhibitory drug binding site in stromelysin and/or the proteoglycan substrate. It is also possible that SP54 and Arteparon increase the stability of collagenase and decrease the stability of stromelysin (in a concentration-dependent manner) during the assay period. However, for collagenase at least, the effects of both drugs were not changed when a shorter (4 hr) assay period was used (data not shown).

Possible in vivo/*clinical significance*

The inhibition of stromelysin activity by SP54 and Arteparon *in vitro* may be of significance to the *in vivo* situation, since clinical doses of Arteparon can produce synovial fluid and serum concentrations of Arteparon [2] spanning the concentrations shown to be inhibitory in Fig. 2. Inhibition of proteoglycan degradation would contribute to chondroprotection *in vivo*.

It is unclear whether the enhancement of collagenase activity by SP54 and Arteparon might have any significance *in vivo*. Indeed, enhancement of collagenase activity is an unexpected property for drugs with chondroprotective activity. However, given that highly sulphated polysaccharides can also alter collagen fibril morphology [17, 21], the effects of SP54 and Arteparon on collagen catabolism *in vivo* are likely to be complex.

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