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EFFECTS OF 1-HYDROXYETHYLIDENE-1,1 BISPHOSPHONATE AND (CHLORO-4 PHENYL) THIOMETHYLENE BISPHOSPHONIC ACID (SR 41319) ON THE MONONUCLEAR CELL FACTOR-MEDIATED RELEASE OF NEUTRAL PROTEINASES BY ARTICULAR CHONDROCYTES AND SYNOVIAL CELLS

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Abstract—Articular chondrocytes and synovial cells were stimulated to produce collagenase, neutral casein and proteoglycan-degrading proteinases by conditioned medium from human peripheral blood mononuclear cells. Collagenase, neutral casein and proteoglycan-degrading proteinase secretion was inhibited by SR 41319, a new bisphosphonate, in a concentration-dependent manner. Complete inhibition was achieved at about 0.3 mM. EHDP exhibited the same general profile but was about 10-fold less active and never completely inhibited the enzyme secretion. When added before MCF, SR 41319 had a protective effect against subsequent activation of the cells by MCF. SR 41319 also inhibited the increase of enzyme secretion by cells previously stimulated with MCF.

The results suggest that the ability of SR 41319 to inhibit the MCF-mediated secretion of neutral enzymes involved in cartilage destruction could be valuable in the management of connective tissue damage in rheumatoid arthritis.

The progressive destruction of joint structure, particularly cartilage, is now recognized as a fundamental feature of chronic inflammatory diseases such as rheumatoid arthritis. Human blood mononuclear cells release a factor which stimulates chondrocytes and synovial cells to produce neutral connective tissue-degrading proteinases [1–6]. This mononuclear cell factor is specifically secreted by monocytes and is identical or closely related to interleukin-1 [1, 2, 7]. Its contribution to the pathogenesis of rheumatoid arthritis is likely as Il-1-like factor has been detected in joint effusions [8, 9] and also probably in serum [10] from patients with active rheumatoid arthritis. Thus, inhibition of MCF†-mediated secretion of neutral proteinases appears as a potential goal for new anti-rheumatic drugs.

Non-steroidal anti-inflammatory drugs and slow acting anti-rheumatic drugs at non-cytotoxic doses display little or no inhibitory effect on MCF-mediated secretion of neutral proteinases [11–13]. In contrast, steroidal anti-inflammatory drugs are very effective [11–13] and, in a preliminary communication, McGuire *et al.* [14] briefly reported that some bisphosphonates (EHDP, Cl2MDP, APD) also inhibit the MCF-mediated production of collagenase by chondrocytes and synovial cells. These compounds are known to exhibit anti-arthritic activity in the model of rat adjuvant arthritis [15–17]. SR 41319 (Fig. 1), a new bisphosphonic compound [18] now under clinical evaluation, also displayed potent anti-arthritic activity in this model [19].

In the present paper, the inhibitory effect of SR 41319 on the MCF-mediated secretion of neutral proteinases by connective tissue cells is reported and compared to a known bisphosphonate, EHDP.

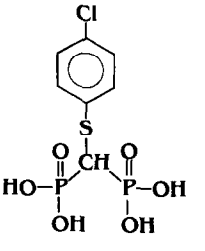

Fig. 1. Structure of (chloro-4 phenyl) thiomethylene bisphosphonic acid (SR 41319).

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† Abbreviations: MCF, mononuclear cell factor; SR 41319, (chloro-4 phenyl) thiomethylene bisphosphonic acid; EHDP, 1-hydroxyethylidene-1,1 bisphosphonic acid, disodium salt; Cl2MDP, disodium dichloromethylene bisphosphonate; APD, 3-amino-1-hydroxypropylidene-1,1 bisphosphonate; DMEM, Dulbecco's Eagle modified medium; PBS, Dulbecco's phosphate buffered saline without Ca and Mg; FCS, fetal calf serum; MCCM, mononuclear cell conditioned medium; Il-1, interleukin-1; TIMP, tissue inhibitor of metallo-proteinases; SD, standard deviation.

SR 41319 and, to a lesser extent, EHDP inhibited the production of collagenase, neutral casein and neutral proteoglycan-degrading proteinases by MCF-stimulated chondrocytes and synovial cells.

MATERIALS AND METHODS

Cell culture techniques

All cell cultures were performed in DMEM (Boehringer, Mannheim, F.R.G.) containing 4.5 g/l glucose, 10^5 i.u./l penicillin and 100 mg/l streptomycin. FCS was heat-inactivated at 56° for 30 min and was acid-treated to inactivate proteinase inhibitors [20]. Cells were cultured at 37° in a humidified 5% (v/v) CO_2–95% (v/v) air atmosphere.

Slices of normal cartilage were taken in aseptic conditions from the femoral and tibial condyles of young rabbit (New Zealand, Charles River, Elbeuf, France) knee joints. Chondrocytes were isolated by digestion (18 hr, 37°) with 0.2% (w/v) bacterial collagenase (Type CLS, Worthington Biochemical Corp., Freehold, N.J., U.S.A.) in DMEM supplemented with 5% (v/v) FCS. The cells were pelleted by centrifugation at 400 *g* for 10 min and washed three times in PBS (Boehringer, Mannheim, F.R.G.). Chondrocytes were plated at a density of 10^5 cells/cm^2 in 175 cm^2 plastic flasks (Nunclon Delta, Roskilde, Denmark) and cultured in DMEM containing 10% (v/v) FCS. The cultures were refed with fresh culture medium every two days and the cells were passaged twice at 10 day intervals using 0.05% (w/v) trypsin–0.02% (w/v) EDTA (Boehringer).

Adherent synovial cells from rabbit knee joints were prepared according to the method of Dayer *et al.* [21]. The cells were plated at a density of 10^6 cells/cm^2 in 175 cm^2 plastic flasks and cultured in DMEM supplemented with 10% (v/v) FCS. The cultures were maintained with changes of medium every 2 days and the cells were passaged twice at 7 day intervals, using 0.05% (w/v) trypsin–0.02% EDTA.

Conditioned medium from cultured human blood mononuclear cells of normal donors was prepared by fractionation on Ficoll-Paque (specific gravity: 1.079) (Pharmacia Fine Chemicals Co., Uppsala, Sweden) at 20°, according to the method of Dayer *et al.* [22]. Non-adherent and adherent mononuclear cells (1×10^6–2×10^6 cells/ml) were cultured for 5 days in DMEM containing 5% (v/v) FCS and 10 μg/ml Pokeweed mitogen (Sigma Chemical Co., St. Louis, MO, U.S.A.). MCCM used as the source of the mononuclear cell factor was filtered on 0.22 μm membrane (Millipore, Bedford, MA, U.S.A.) and stored at −20° until use.

Stimulation of chondrocytes and synovial cells with MCF and investigation of drug effects were carried out on cells which had been subcultured in 16 mm multiwell plates (Nunclon Delta). Chondrocytes and synovial cells were plated at a density of 1×10^5 cells/cm^2 and 5×10^4 cells/cm^2, respectively and each well contained 2 ml DMEM supplemented with 10% (v/v) FCS. At confluence, the medium was replaced with MCCM, diluted 1:5 with DMEM. Drugs to be assayed were added at the same time. Controls containing only DMEM supplemented with an equivalent amount of FCS (1% (v/v)) and pokeweed mitogen (2 μg/ml) were run in parallel to allow evaluation of the basal level of enzyme production by cells. After 72 hr incubation, the media were harvested and stored at −20° until assayed. Cell viability was checked by the ability of cells to exclude trypan blue stain. In some experiments, these standard culture conditions were modified as follows. (1) The cells were first activated with MCF for 48 hr, then washed with PBS and cultured for 72 hr in DMEM supplemented with 1% (v/v) FCS and the drugs under investigation. (2) The cells were first cultured in DMEM containing 1% (v/v) FCS and the drugs under investigation for 48 hr, then washed with PBS and cultured in MCCM (diluted as above) for 72 hr. To evaluate possible drug effects on proteoglycan and collagen biosynthesis, confluent cell cultures were refed with 2 ml DMEM supplemented with the drug under investigation, 10% (v/v) FCS and either 10 μCi/ml ^{35}S-sulphate (Amersham, Versailles, France) or 1 μCi/ml ^{14}C-proline. After 72 hr incubation, the media containing the macromolecules secreted by the cells were harvested and stored at −20° until assay.

Drugs

SR 41319 was from Sanofi Research (Clin-Midy Research Center, Montpellier, France). 1-Hydroxyethane-1,1 bisphosphonic acid, disodium salt (Etidronate, Procter & Gamble Co., Cincinnati, OH, U.S.A.) was obtained from Ricerchimica (Milano, Italy). The drugs were dissolved in PBS and filter-sterilized using 0.22 μm membranes.

Assays

Neutral collagenase assays were done on soluble native ^{14}C-labelled collagen at 25° by the method of Vaes [23], but using collagen labelled with ^{14}C-acetic anhydride [24]. Neutral casein-degrading activity was assayed at pH 7 on tritium-labelled casein, according to the method of Vaes *et al.* [25]. Cartilage proteoglycan-degrading activity at neutral pH and 37° was measured by the release of ^{3}H-soluble material from polyacrylamide beads containing ^{3}H-labelled proteoglycan as described by Dingle *et al.* [26]. Proteoglycan aggregates were prepared from bovine nasal cartilage and ^{3}H-labelled as previously described [27]. All enzymic assays were performed after culture medium treatment with trypsin (Type TPCK, Worthington Biochemical Corp.) (100 μg/ml culture medium) for 30 min at 25° and then with soybean trypsin inhibitor (Sigma Chemical Co.) (400 μg/ml culture medium), to convert any latent enzymes (proenzymes and/or complexes of enzyme-inhibitor) to their active forms [22, 23, 28–31]. Proteoglycan biosynthesis was determined by measuring the ^{35}S-sulphate specifically incorporated in their glycosaminoglycan moieties as described by Saarni and Tammi [32]. ^{14}C-Proline specifically incorporated into collagen was evaluated to quantify collagen biosynthesis, according to the method of Jalkanen *et al.* [33].

Statistical analysis

All results are expressed as the mean ± standard

Table 1. Neutral proteinase activities after exposure of chondrocytes and synovial cells to MCF*

	Neutral proteinase activities		
	Collagenase (Units/dish)	Casein-degrading (Units/dish)	Proteoglycan-degrading (Units/dish)
(1) Articular chondrocytes			
(i) control	0.1 ± 0.1	0.20 ± 0.05	2 ± 1
(ii) +MCF	45 ± 3†	9.2 ± 0.7†	22 ± 2†
(2) Synovial cells			
(i) control	1.0 ± 0.6	0.6 ± 0.3	3 ± 1
(ii) +MCF	52 ± 1†	9.1 ± 0.7†	17 ± 1†

* Cells at confluence were cultured for 3 days in 2 ml of MCCM diluted 1:5 with DMEM. The media were then harvested and the enzymic activities were determined. The units of collagenase and casein-degrading enzyme have been previously defined [19, 21]. One unit of proteoglycan-degrading enzyme refers to the amount of enzyme that degrades 1% of substrate/min during an incubation of 2 hr at 37°. Each result is the mean of three cultures ± S.D.

† $P < 0.01$ in the Student's t-test, comparing MCF-stimulated and unstimulated cells.

deviation, and comparisons were analysed by the Student's t-test for significance.

RESULTS

Conditioned medium of human mononuclear cells, containing the mononuclear cell factor, stimulated normal chondrocytes and synovial cells to produce large amount of collagenase, neutral casein and proteoglycan-degrading proteinases while unstimulated cells released very low amounts of enzymes into the medium (Table 1). The effect of SR 41319 is shown in Figs 2–4. Simultaneous MCF-stimulation and SR 41319 treatment of the cells greatly reduced the production of neutral casein-degrading enzyme (Fig. 2) and collagenase (Fig. 3). Proteoglycan-degrading activity was also inhibited by SR 41319 (Fig. 4). The action of SR 41319 was similar in cultures of

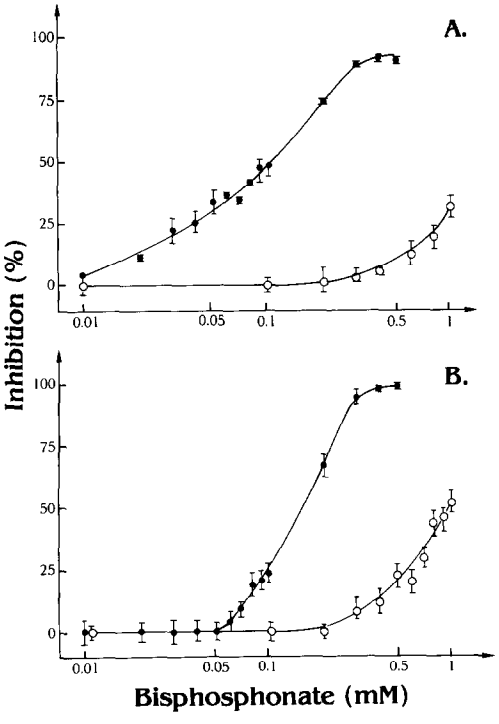

Fig. 2. MCF-mediated secretion of casein-degrading proteinase by synovial cells (A) and chondrocytes (B). Cells were cultured for 72 hr in MCCM diluted 1:5 with DMEM, and treated simultaneously with varying concentrations of SR 41319 (●) and of EHDP (○). Enzymic activities of controls are given in Table 1. Results are expressed as % inhibition ± S.D. (N = 3) and the doses are on a logarithmic scale.

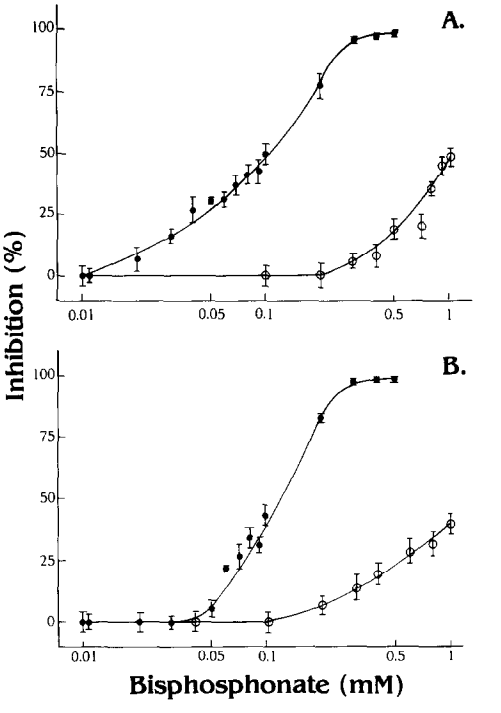

Fig. 3. MCF-mediated secretion of collagenase by synovial cells (A) and chondrocytes (B). Cells were cultured for 72 hr in MCCM diluted 1:5 with DMEM, and treated simultaneously with varying concentrations of SR 41319 (●) and of EHDP (○). Enzymic activities of controls are given in Table 1. Results are expressed as % inhibition ± S.D. (N = 3) and the doses are on a logarithmic scale.

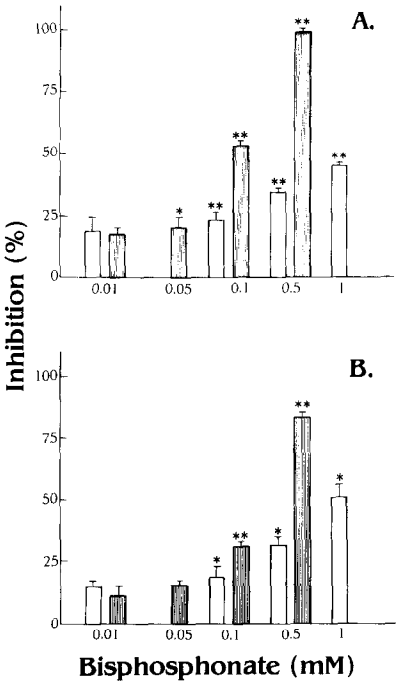

Fig. 4. MCF-mediated secretion of proteoglycan-degrading proteinase by synovial cells (A) and chondrocytes (B). Cells were cultured for 72 hr in MCCM diluted 1:5 with DMEM, and treated simultaneously with varying concentrations of SR 41319 (■) and of EHDP (□). Enzymic activities of controls are given in Table 1. Results are expressed as % inhibition ± SD (N = 3). *P < 0.05, **P < 0.01 comparing drug treatment with MCF-stimulated controls.

chondrocytes and synovial cells. All these effects were concentration-dependent over a range of 0.01–0.5 mM and complete inhibition was achieved at the highest doses. This concentration range is in agreement with results obtained in other studies on different biochemical effects of bisphosphonates in various cell systems [34–38]. EHDP had the same general profile as SR 41319 but was much less active (Figs 2–4); active concentrations were in the range of 0.1–1 mM and complete inhibition was never observed.

If chondrocytes and synovial cells were treated with SR 41319 after MCF-stimulation, the drug still inhibited the increased secretion of casein-degrading proteinase induced by the previous MCF-stimulation (Fig. 5). This inhibition was concentration-dependent and was almost complete at the highest concentrations. EHDP also inhibited under these conditions but was again less active than SR 41319. These experiments suggest that the drug effects did not depend on the simultaneous addition of MCF and the drug.

Preincubation of chondrocytes and synovial cells for 48 hr with SR 41319 followed by removal of the drug also prevented subsequent activation of the cells by MCF, as demonstrated by the reduced secretion of casein-degrading proteinase (Fig. 6). The effectiveness of SR 41319 was a little weaker, but still significant. Shorter preincubation time led to a weak and insignificant inhibition. These experiments suggest that intracellular accumulation of the drug was probably essential to exert a protective action against MCF-stimulation, and that such intracellular drug concentrations were reached slowly and needed high extracellular concentrations of the drugs. This interpretation is consistent with the observations that the uptake of labelled EHDP and Cl2MDP by chondrocytes and fibroblasts was very slow and was dependent on the extracellular concentration and on the time of exposure [34]. These results also confirm the lack of correlation between the drug activities and the schedule of MCF-stimulation and drug treatment.

Chondrocytes and synovial cells have been shown to synthesize a tissue inhibitor of metallo-proteinases [11, 12, 39, 40] and some drugs are able to increase TIMP synthesis [11, 12, 40]. The possible effect of SR 41319 and EHDP on the TIMP synthesis remains to be established. However, trypsin treatment of culture media was always performed under optimal conditions to convert any latent enzymes (in particular, complexes of enzyme-inhibitor) to their active forms [23, 28–31]. It is thus unlikely that TIMP could

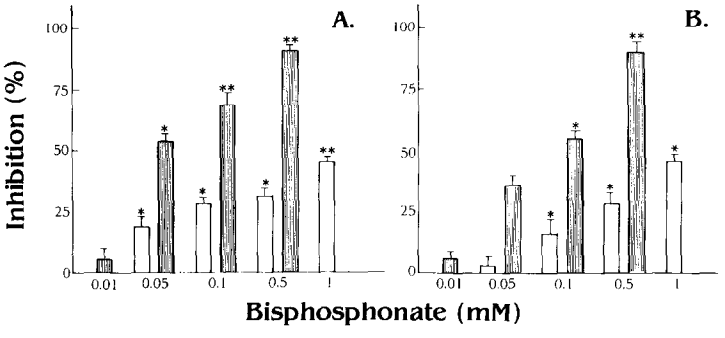

Fig. 5. Effect of SR 41319 (■) and of EHDP (□) on the secretion of casein-degrading proteinase by synovial cells (A) and chondrocytes (B) previously stimulated with MCF. Cells were cultured for 48 hr in MCCM diluted 1:5 with DMEM, washed with PBS and then cultured for 72 hr in DMEM containing varying concentrations of drug. Enzymic activities of synovial cell controls were 0.3 ± 0.1 and 5.5 ± 0.1 while those of chondrocytes were 0.015 ± 0.05 and 6.6 ± 0.2 respectively for unstimulated and MCF-stimulated cells. Results are given as % inhibition ± S.D. (N = 3). *P < 0.05, **P < 0.01 comparing drug treatment with MCF-stimulated controls.

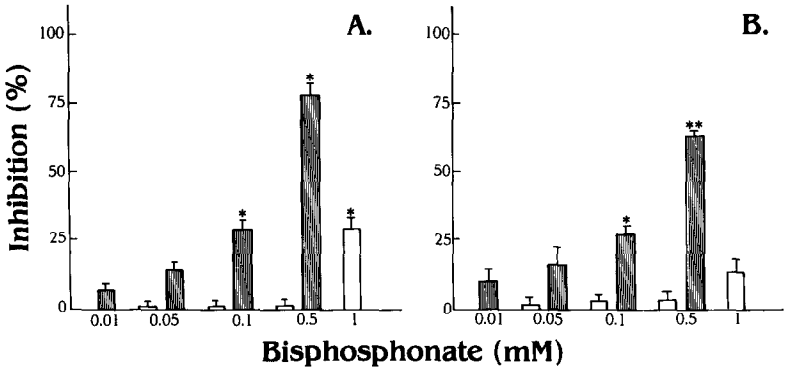

Fig. 6. Protective effect of SR 41319 (■) and of EHDP (□) against MCF-stimulation of synovial cells (A) and chondrocytes (B). Secretion of casein-degrading proteinase. Cells were cultured for 48 hr in DMEM containing varying concentrations of drug, washed with PBS and then cultured for 72 hr in MCCM diluted 1:5 with DMEM. Enzymic activities of synovial cell controls were 0.35 ± 0.05 and 6.2 ± 0.4 while those of chondrocytes were 0.9 ± 0.1 and 9.2 ± 0.6 respectively for unstimulated and MCF-stimulated cells. Results are given as % inhibition ± S.D. (N = 3). *P < 0.05, **P < 0.01 comparing drug treatment with MCF-stimulated controls.

interfere in the determination of enzymic activities, whatever its level may be.

The decrease of enzyme secretion was not related to SR 41319 or EHDP cytotoxicity since exclusion of trypan blue (used to test cell viability of the culture) was always demonstrated. The activity of SR 41319 and EHDP was not associated with a general, non-specific inhibition of cellular biosynthetic processes, since preliminary experiments showed that biosynthesis of collagen and proteoglycan was not significantly affected by the drugs (Table 2). These observations are in line with reported data on EHDP and Cl2MDP, which show that biosynthesis of collagen and proteoglycan by chondrocytes was not affected by EHDP and was actually increased by Cl2MDP [35, 36]. SR 41319 (0.5 mM) and EHDP (1 mM) did not directly inhibit collagenase and neutral proteinases in the enzymic assays.

Table 2. Incorporation of ^{35}S-sulphate into proteoglycan and ^{14}C-proline into collagen synthesized by chondrocytes and synovial cells*

	Radioactivity incorporated (CPM/dish)	
	Proteoglycan	Collagen
(1) Articular chondrocytes		
(i) control	305,928 ± 2276	68,680 ± 8475
(ii) SR 41319		
0.05 mM	297,644 ± 11,512	64,495 ± 11,530
0.1 mM	308,832 ± 7832	61,435 ± 5000
0.5 mM	303,392 ± 15,420	66,205 ± 6765
(iii) EHDP		
0.1 mM	309,346 ± 540	63,345 ± 5450
0.5 mM	308,996 ± 11,827	68,425 ± 8305
1 mM	286,796 ± 10,544	71,935 ± 1365
(2) Synovial cells		
(i) control	113,328 ± 3215	114,055 ± 8527
(ii) SR 41319		
0.05 mM	112,736 ± 7252	102,495 ± 11,135
0.1 mM	114,328 ± 6932	114,020 ± 9159
0.5 mM	112,872 ± 8324	113,615 ± 10,828
(iii) EHDP		
0.1 mM	116,692 ± 2600	117,360 ± 7537
0.5 mM	114,188 ± 1600	110,520 ± 6545
1 mM	108,176 ± 6000	107,360 ± 13,964

* Cells at confluence were cultured for 72 hr in 2 ml DMEM containing the drug under investigation, 10% (v/v) FCS and either 10 μCi/ml ^{35}S-sulphate or 1 μCi/ml ^{14}C-proline.

The results represent radioactivity incorporated into proteoglycans and collagen released into the media and are expressed as means ± S.D. (three cultures). No significant differences were obtained in the Student's *t*-test, comparing bisphosphonate-treated and controls.

DISCUSSION

Our studies have demonstrated that SR 41319, a new bisphosphonate, inhibited the MCF-mediated secretion of collagenase, casein and proteoglycan-degrading proteinases by chondrocytes and synovial cells. A single treatment of these cells with SR 41319 at concentrations between 0.01 and 0.5 mM induced a concentration-dependent inhibition of the MCF-stimulated secretion of neutral proteinases. Complete inhibition was reached at the highest concentrations without cell cytotoxicity. SR 41319 also inhibited, in a concentration-dependent manner, increased enzyme secretion by cells previously activated by MCF. Moreover, preincubation of the cells with SR 41319 prevented subsequent activation by MCF.

Compared to SR 41319, EHDP was much less active and, unlike SR 41319, complete inhibition was never observed at non-cytotoxic concentrations. In preliminary communications, McGuire *et al.* [14, 41] have reported that Cl2MDP and APD are more active than EHDP at inhibiting MCF-mediated production of collagenase by chondrocytes and synovial cells. It should, however, be emphasized that these results were obtained with growing cells after 12–14 days of cell exposure to the drugs. Further experiments are needed in order to compare SR 41319 with other bisphosphonates.

SR 41319 showed a biochemical profile clearly different from that of non-steroidal anti-inflammatory and anti-rheumatic drugs, since the latter, at non-cytotoxic concentrations, did not inhibit the MCF-mediated secretion of neutral proteinases by chondrocytes and synovial cells [11–13]. Steroidal anti-inflammatory drugs completely inhibited the MCF-mediated secretion of neutral proteinases [11–13], while retinoids had a partial inhibitory effect [11], and both were effective at much lower concentrations than SR 41319. Razoxane, an anti-psoriatic drug used in the treatment of psoriatic arthritis, was also found to inhibit collagenase secretion by MCF-stimulated chondrocytes at concentrations between 0.1 and 1 mM. This effect was, however, clearly demonstrated only against mild MCF stimulation after repeated daily treatment of the cells with razoxane [40].

The mechanism of SR 41319 inhibition of MCF-stimulated secretion of neutral proteinases is not fully understood. SR 41319 could either prevent the interaction between cells and the MCF-protein or inhibit the MCF-induced biosynthetic processes responsible for the increased synthesis and/or secretion of neutral proteinases. As we observed SR 41319 activity even if MCF and drug treatments were not simultaneous, the second hypothesis seems more likely. This intracellular inhibitory process is not, however, a general biosynthetic inhibition since no effects of SR 41319 were observed on the biosynthesis of collagen and proteoglycan. The *in vitro* activity of SR 41319 was obtained at relatively high concentrations, but bearing in mind that bisphosphonates [42], and especially SR 41319, are strongly bound to bone and cartilage *in vivo* (W. Cautreels *et al.*, unpublished results) while only low plasma concentrations were achieved, it is not unreasonable to consider, as already pointed out by Fast *et al.* [34], that high local concentration of bisphosphonates could be reached *in vivo* when the sequestered drug is released during bone and cartilage turnover.

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