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THE EFFECT OF ANTIRHEUMATIC DRUGS ON COLLAGENOLYTIC ACTIVITY OF CATHEPSIN B1

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Abstract—Seven antirheumatic drugs were tested for their possible inhibition of collagenolytic activity of cathepsin B1. Arteparon, Antilysin, Sanocrysin and phenylbutazone were found to be potent inhibitors of collagenolytic activity of cathepsin B1. Indomethacin and flufenamic acid were weak inhibitors, flurbiprofen was ineffective. A possible mechanism of action is discussed.

Lysosomal enzymes play an important role in the pathogenesis of rheumatic diseases and in the degradation of the connective tissue. One of the enzymes is cathepsin B1 a thiol-dependent protease of lysosomal origin with a broad spectrum of specificity. It degrades the synthetic substrates Bz-Arg-NH_2, Bz-DL-Arg-$NHC_6H_4NO_2$, Bz-DL-Arg-$NHC_{10}H_7$, Bz-Arg-OET at optimum pH 6.0 [1]. Proteoglycanes and collagen are important natural substrates of cathepsin B1. Cathepsin B1 degrades the protein core of proteoglycans in a similar way as does papain [2]. It degrades native collagen in solution in the presence of EDTA and cysteine the pH optimum being 4.5–5.0. The initial action at 24° is cleavage of collagen molecule in its non-helical region containing cross-links. The enzyme also attacks the helical region of collagen and low molecular weight peptides occur [3]. The mechanism of collagenolytic activity of cathepsin B1 differs substantially from that of mammalian neutral collagenase which degrades the collagen molecule first in its helical part into two discrete fragments [4]. Cathepsin B1 degraded also insoluble collagen with a pH optimum below 4 and *in vitro* reconstituted collagen fibrils with a pH optimum about 5.6 [3].

It seems the cathepsin B1 together with collagenolytic cathepsin are responsible for the collagenolytic activity of lysosomal extract at acidic pH [5]. They play an important role in intracellular degradation of collagen although their extracellular activity cannot be excluded.

Kruze *et al.* [6] have studied the effects of antirheumatic drugs with the synthetic substrate Bz-DL-Arg-$NHC_{10}H_7$ on the activity of cathepsin B1. They found out that there are several antirheumatic drugs that inhibit the activity of cathepsin B1. Our work was aimed at the study of the effects of antirheumatic drugs on the collagenolytic activity of cathepsin B1.

Abbreviations used: Bz-Arg-NH_2, α-*N*-benzoyl-L-arginine-amide; Bz-DL-Arg-$NHC_6H_4NO_2$, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; Bz-DL-Arg-$NHC_{10}H_7$, α-*N*-benzoyl-DL-arginine 2-naphthylamide; Bz-Arg-OET, α-*N*-benzoyl-L-arginine ethyl ester.

MATERIALS AND METHODS

Isolation and purification of cathepsin B1. Cathepsin B1 was isolated from bovine spleen by the method Keilová and Tomášek [7]. The final purification of the enzyme was achieved by affinity chromatography on a mercurial Sepharose column. Enzyme from the inactive mercury form was released with dithioerythreitol and the free mercury was removed by ultrafiltration through an UM-10 Amicon membrane.

The enzymatic activity of cathepsin B1 was determined in terms of hydrolysis of Bz-DL-Arg-$NHC_6H_4NO_2$, as substrate using a procedure described by Keilová and Tomášek [7]. The quantity of the enzyme which cleaved 1 μmole of a substrate in 1 min under the conditions described was taken for one unit (U).

Substrate preparation. The acid-extracted radioactive collagen was prepared after injecting 40-day-old Wistar rats intraperitoneally with uniformly labelled [^{14}C-]glycine (70 mCi/mmole). Each rat received 50 μC of isotope 72, 60, 48, 24 and 12 hr before sacrifice [8]. The acid-extractable collagen was purified as described by Kang *et al.* [9]; it had radioactivity 22.000 d.p.m./mg.

The reconstituted ^{14}C-labelled collagen fibrils were prepared by heating 2 mg/ml solutions of collagen containing 50 mM Tris–HCl buffer, pH 7.4 with 0.2 M NaCl at 35° overnight. The reconstituted fibrils were incubated with trypsin to check that the collagen had not been denatured during preparation and with clostridial collagenase to measure total fibril lysis [10].

Assay of collagenolytic activity of cathepsin B1. The collagen fibrils (approx. 0.75 mg collagen) were resuspended in the 0.9 ml 0.1 M phosphate buffer of pH 6 containing 1 mM EDTA (disodium salt) with or without drugs by shaking. 12 mU of cathepsin B1 was preincubated in the 0.1 ml phosphate buffer containing 6 mM dithioerythreitol 20 min at the room temperature. After the enzyme was added to the substrate the reaction mixture was incubated at 35° for 20 hr. Controls with phosphate buffer containing dithioerythreitol in place of enzyme and blanks containing appropriate buffers without drugs were also

included and the pH of each reaction mixture was checked.

At the end of the incubation period the unchanged collagen was removed by centrifugation 10,000 *g* for 20 min and the radioactivity released was measured as described by Werb and Burleigh [10]. Results are expressed as a percentage of total fibril lysis after subtraction of the buffer control values. The fibril lysis in the presence and absence of drugs was compared.

Drugs. The following drugs were were used in the experiments: indomethacin (Indren), phenylbutazone (Fenylbutazon), inhibitor of proteases from lung (Antilysin-polypeptide of molecular weight 6511) all manufactured by Spofa, Czechoslovakia; flurbiprofen (2-(fluorobiphenylyl)propionic acid) from Boots, England; sodium aurothiosulphate (Sanocrysine) produced by Ferrosan, Denmark; flufenamic acid from Parke and Davis, England and Arteparon (glycosaminoglycan polysulfate) from Luitpold Werk, West Germany.

Drugs not soluble in 0.1 M phosphate buffer (indomethacin, phenylbutazone, flurbiprofen and flufenamic acid) were first dissolved in a small volume of dimethyl sulphoxide (1%).

RESULTS AND DISCUSSION

The effect of antirheumatic drugs on the collagenolytic activity of cathepsin B1 is presented in Table 1. Phenylbutazone, Antilysin, Arteparon and Sanocrysine were the most potent inhibitors. Indomethacin, flufenamic acid were weak inhibitors, flurbiprofen had no inhibitory effect.

In the group of nonsteroidal antirheumatic drugs the most distinct inhibitory effect on the collagenolytic activity of cathepsin B1 showed phenylbutazone. This result is in agreement with the observation that phenylbutazone inhibits the collagenolytic activity of lysosomal extract at acid pH *in vitro* [11] and decreases the collagenolytic activity in inflamed tissues *in vivo* [12, 13] as well. The inhibitory effect of phenylbutazone on the enzymatic activity of cathepsin B1 with low-molecular substrate Bz-DL-Arg-$NHC_{10}H_7$ was described by Kruze, Fehr and Böni [6]. It is known that phenylbutazone as an acidic drug is able to interact with basic groups of proteins [14]. It is therefore quite possible that the inhibition of the collagenolytic activity of cathepsin B1 with phenylbutazone results from its interaction both with collagen and cathepsin B1.

Indomethacin inhibits the collagenolytic activity of cathepsin B1 about 50 per cent less than phenylbutazone. This drug also shows an inhibitory activity when Bz-DL-Arg-$NHC_{10}H_7$ is used as substrate [6]. Other nonsteroidal antirheumatic drugs such as flufenamic acid and flurbiprofen showed only weak or no effects on the collagenolytic activity of cathepsin B1.

The tissue extracts in our group of tested antirheumatic drugs were represented by Arteparon and Antilysin. Table 1 and Fig. 1 showed that Arteparon is a very potent inhibitor of collagenolytic activity of cathepsin B1. A 60 per cent inhibition could be observed with phenylbutazone and Antilysin in 1 mM concentration whereas Arteparon had the same inhibitory activity in a concentration 250 times lower (4 μM).

Arteparon contains sulfonated glycosaminoglycan chains and it has favourable effects particularly in the case of degenerative joint diseases. Arteparon inhibits other lysosomal enzymes taking part in dissociation of mucopolysaccharides of cartilage, such as β-glucuronidase, β-galactosidase, etc. [15].

Dettmer [16] described the binding of Arteparon on the surface of collagen fibrils and cumulation of this drug in the cartilage. The interaction between Arteparon and the collagen fibrils has been proved also by our finding that Arteparon prevents the collagen fibrils from dissolving at pH 6 (Fig. 1). It is known that the collagen fibrils dissolve spontaneously

Table 1. Effect of antirheumatic drugs on collagenolytic activity of cathepsin B1

Drugs	Concentration (mM)	Inhibition (%)
Phenylbutazone	0.5	22.5
	1.0	57.2
Indomethacin	0.5	9.4
	1.0	22.1
Flufenamic acid	0.5	5.1
	1.0	14.1
Flurbiprofen	0.5	0
	1.0	0
Antilysin	0.5	30.0
	1.0	63.0
Arteparon	0.5	100.0
	1.0	100.0
Sanocrysine*	0.5	53.1
	1.0	81.0

The assay system contained 0.9 ml of a suspension of reconstituted collagen fibrils (0.75 mg collagen) in a 0.1 M phosphate buffer pH 6 containing 1 mM EDTA and drugs and 0.1 ml of enzyme solution (12 mU of enzyme).

* The concentration of EDTA was 0.5 mM when Sanocrysine was assayed.

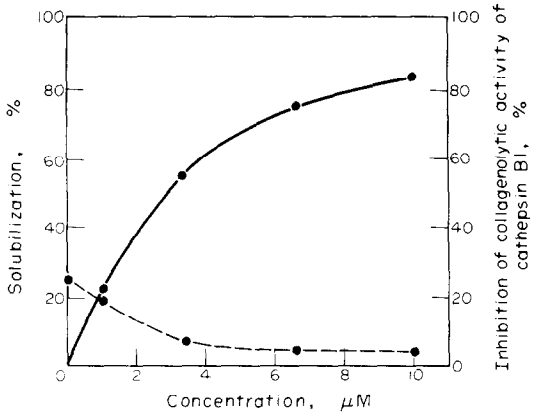

Fig. 1. Effect of different concentrations of Arteparon on the collagenolytic activity of cathepsin B1 ——, and on the solubilization of the collagen fibrils at pH 6 in the absence of enzyme – – –.

in an acidic medium. While in the case of controls as much as 23 per cent of the collagen fibrils dissolved at 35°, only 4 per cent of the collagen fibrils dissolved in the presence of Arteparon in 10 μM concentration (Fig. 1). It is therefore quite probable that the inhibition of the collagenolytic activity of cathepsin B1 with Arteparon is due to its effects on both the substrate and the enzyme.

Antilysin is a polypeptide inhibitor isolated from bovine lungs. It inhibits kallikrein and some serine proteases [17]. It was found to be a strong inhibitor of the collagenolytic activity of cathepsin B1 but without any affects on dissolving of the collagen fibrils.

Sanocrysin was after Arteparon the most active inhibitor of collagenolytic activity of cathepsin B1. There was an 81 per cent inhibition of the cathepsin B1 in the presence of this gold containing drug in 1 mM concentration (Table 1). It is probable that gold as a heavy metal could block the thiol group in the active centre of the enzyme. Gold is known to inhibit the activities of several lysosomal enzymes, such as human cathepsin B1 [18], β-glucuronidase, acidic phosphatase [19] as well as leucocyte collagenase [20].

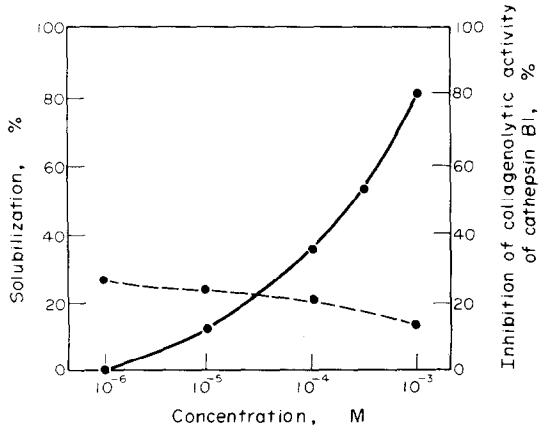

Fig. 2. Effect of different concentrations of Sanocrysine on the collagenolytic activity of cathepsin B1 ——, and on the solubilization of the collagen fibrils at pH 6 in the absence of enzyme – – –.

The results of our experiment showed further that Sanocrysin also stabilises the collagen fibrils *in vitro* at pH 6 preventing their dissolution (Fig. 2).

The inhibitory effects of some tested antirheumatic drugs on the collagenolytic activity of cathepsin B1 could be partially explained by the interaction of these drugs and the substrate. Considering the experiments carried out by Kruze *et al.* [6] who found the inhibition of the hydrolytic activity of cathepsin B1 with these drugs when using the synthetic substrate Bz-DL-Arg-$NHC_{10}H_7$, one cannot exclude their dirrect effect on the enzyme. It is therefore quite possible that in the course of the inhibition of the collagenolytic activity of cathepsin B1 both mechanisms are operating.

It is difficult to discuss the results from the standpoint of clinical therapy. Although some of the used concentrations of tested drugs are higher than the therapeutic blood levels we must point out the difference between the concentrations in blood and in the target tissue. It appears that especially acidic nonsteroidal antirheumatic drugs accumulate specifically in inflamed tissue [21] and therefore attained concentrations of these drugs could be high enough to inhibit cathepsin B1 *in vivo*. As some of the antirheumatic drugs accumulate significantly in lysosomes their intracellular inhibitory effect on cathepsin B1 could not be ruled out [22].

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