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REVIEW ARTICLE

COLLAGENASE INHIBITORS: THEIR DESIGN AND POTENTIAL THERAPEUTIC USE

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INTRODUCTION

The aim of this review is to summarise the properties of mammalian collagenases with specific reference to those properties which can be utilised, practically or theoretically, in the design and assessment of inhibitors of collagenases. The current status of collagenase inhibition will be detailed and the ways in which substrate structure and enzyme specificity can be used in inhibitor design will be discussed. The possible role of collagenases in the pathogenesis of human disease and the potential therapeutic value of low molecular weight collagenase inhibitors in those diseases will be considered. While it cannot be discounted that damage to host connective tissue may result from the action of collagenases derived from invasive bacteria such events would best be inhibited by antibacterial agents to eradicate the infection. This review will therefore consider only mammalian collagenases and their inhibition.

COLLAGEN – THE NATURAL SUBSTRATE

The structure of collagen has been reviewed by Miller.[1] It is the major structural protein in mammals. It consists of three left-handed helical polypeptide α-chains, which supercoil around a common axis to form a right-handed triple helix.

In each chain every third residue is a glycyl with prolyl and hydroxyprolyl respectively predominating as the next two residues. The macromolecule (*ca.* 300,000 daltons) is stabilised by interchain hydrogen bonds and further by inter- and intramolecular covalent links. Several different forms of collagen are found. Those designated Types I to IV collectively represent over 95% of the total. This heterogeneity appears to reflect physiological function. Type I forms relatively large, well structured, fibres giving strength and limited extensibility to tissues. It forms the collagen network in bone and tendon. In many other tissues, e.g. skin, arterial wall, uterine

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wall and synovium, Types I and III are found together. Type III forms small fibres organised into rectilinear networks allowing some distensibility and compliance in the tissue.

Type II collagen exists largely in hyaline cartilage and vitreous humour. It forms smaller fibrils than Type I. In conjunction with proteoglycan this collagen forms structures with high resistance to compression. Type IV is found in basement membranes where it appears to provide a supporting network. It is distinguished from Types I to III in that its amino acid sequence contains several areas where the tripeptide repeat unit is interrupted. This may result in increased flexibility of the triple helix.[2]

The individuality of the collagen molecule with its triple helix and high content of proline and hydroxyproline renders it resistant to degradation by most mammalian proteases.

COLLAGENASES

The true collagenases are those enzymes which at physiological pH and 25°C make a single cleavage of native collagen producing approximately three quarter and one quarter fragments (one third and two thirds for Type IV).[3] (Figure 1) At this temperature these fragments are stable and resist further degradation by collagenase and other proteases. However, at physiological temperature (above 33°) these products spontaneously denature and become susceptible to cleavage by gelatinases and a wide range of other less specific proteases. Thus under physiological conditions the collagenases initiate the degradation of the whole macromolecule by effecting one specific cleavage. Control of collagen degradation and turnover is thus critically controlled by the collagenases and hence modulation or inhibition of collagen turnover should be achieved by control of collagenase activity.

It has been suggested[4] that the specific sequence compatible with cleavage by collagenase is either Gly-Ile-Ala or Gly-Leu-Ala. However inspection of the available sequence data for α-chains[5] indicates that the above three amino acid repeat also occurs in other locations of the collagen chain which are not susceptible to collagenase

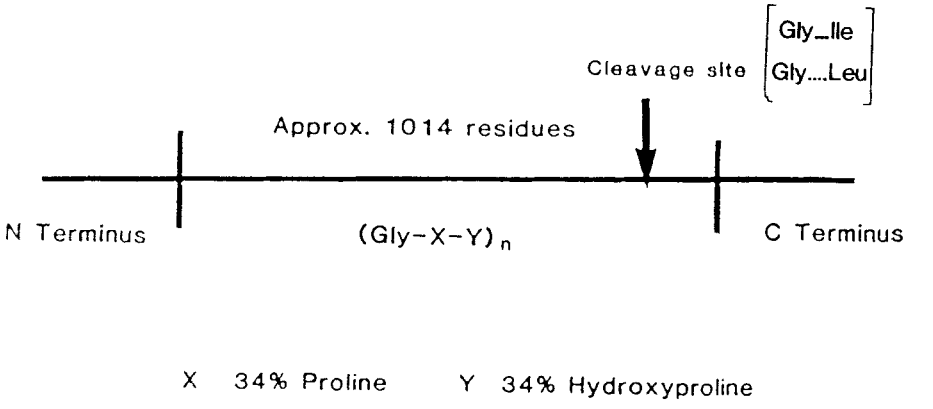


FIGURE 1 Features common to all collagen monomers. The collagenase cleavage site is indicated.

cleavage. A detailed computer-assisted search[6] of all Gly-Leu and Gly-Ile bonds in the primary structure of 1042 residues[5] of chick skin collagen shows that the scissile bond is unique in having adjoining residues conforming with the amino acid sequence shown in Table Ia. Extending this analysis to include collagenase susceptible bonds of collagens from other sources (Table Ib) it can be seen that a minimum of five residues spanning subsites P3 to P2′ represents the substrate recognition site in collagenase.[6] Collagenases from some sources may make additional limited cleavages C-terminal to the main cleavage site. Evidence for this is reviewed in detail by Gross *et al.*[7]

SOURCES AND PROPERTIES OF COLLAGENASES

If, as appears to be the case, collagenases control the turnover, remodelling and degradation of collagen in tissue then where there is collagen there must be the facility to produce collagenase. Evidence to substantiate this generalisation initially proved difficult to obtain. Indeed vertebrate collagenases could not be identified until 1962 when Gross and Lapier[8] detected the enzyme in the serum free culture medium of resorbing tadpole tail tissue. There are several properties of collagenases which explain the initial difficulty in its detection. With one exception collagenase is not stored in cells but synthesised *de novo* on cell stimulation. Collagenases are produced as inactive proenzymes which must be activated by limited proteolysis and/or treatment with mercurials or other agents which react with thiols. Collagenases are inhibited by some serum protease inhibitors, namely α_2-macroglobulin[9] and β_1-anti-collagenase[10] and by a specific inhibitor of metalloproteases (TIMP)[11] often cogenerated in culture with procollagenase.[12] The activation of procollagenases has been reviewed by Murphy and Sellers.[13]

(Pro)collagenase production has now been demonstrated for a large number of cell types in culture. Collagenase production by human cells has been demonstrated by

TABLE Ia
Preferred amino-acid sequence for collagenase cleavage

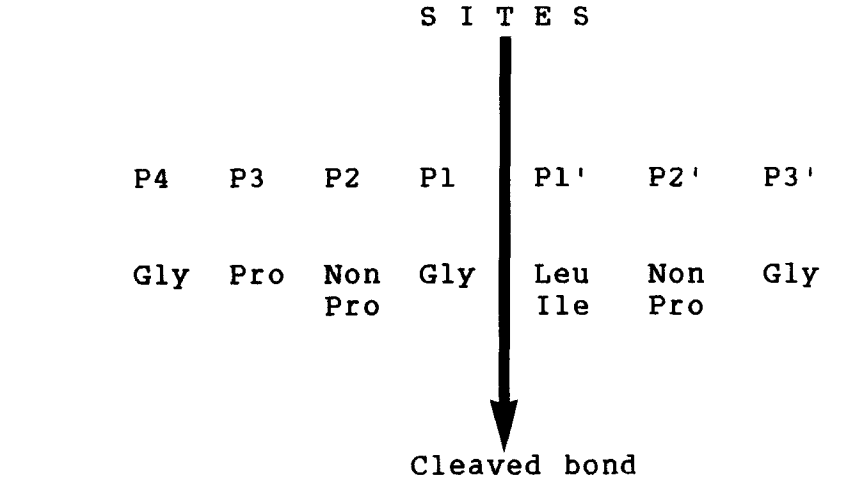


TABLE Ib
Amino acid sequence at the collagenase cleavage site

Source and Collagen Type	Subsite P4 P3 P2 P1 ↓ P1′ P2′ P3′	Reference
Bovine α_1 (I)	Gly-Pro-Gln-Gly Ile-Ala-Gly	Hofmann *et al., J. Mol. Biol.*, **174**, 137 (1978)
Bovine α_2 (I)	Gly-Pro-Gln-Gly Leu-Leu-Gly	Miller E.J., *Extracellular Matrix Biochem.*, p. 41 (1984)
Bovine α_1 (II)	UNKNOWN-Gly Ile-Ala-Gly	Miller *et al., Biochem.*, **15**, 787, (1976)
Bovine α_1 (III)	Gly-Pro-Leu-Gly Ile-Ala-Gly	Miller E.J., *Extracellular Matrix Biochem.*, p. 41 (1984)
Human α_1 (I)	Gly-Pro-Gln-Gly Ile-Ala-Gly	Bernard *et al., Biochem.*, **22**, 5213 (1983)
Human α_1 (II)	UNKNOWN-Gly Ile-Ala-Gly	Miller E.J., *Extracellular Matrix Biochem.*, p. 41 (1984)
Human α_1 (III)	Gly-Pro-Leu-Gly Ile-Ala-Gly	Seyer *et al., Biochem.*, **19**, 1583 (1980)
Chicken α_1 (I)	Gly-Pro-Gln-Gly Ile-Ala-Gly	Highberger *et al., Biochem.*, **21**, 2048 (1982)
Mouse α_1 (I)	Gly-Pro-Gln-Gly Ile-Ala-Gly	Monson *et al., Mol. Cell Biol.*, **2**, 1362 (1982)
Chicken α_2 (I)	Gly-Pro-Gln-Gly Ile-Leu-Gly	Dixit *et al., Biochem.*, **18**, 5416 (1979)

synovial[14] and skin[15] fibroblasts, chondrocytes,[16] peripheral mononuclear cells,[17] keratinocytes[18] and gingival tissue.[19] Collagenases from a number of human tumour cells preferentially cleave collagen Type IV.[3,20,21] The collagenase of polymorphonuclear leucocytes is distinct in that it is stored, again in latent form, in the specific granules and hence can be extracted directly from those cells.[22]

The collagenases from different cell types and species have markedly different physical properties. Molecular weights reported for the latent enzyme range from 40,000 to 120,000 about 10–20,000 usually being lost on activation.[13] They exhibit a wide range of isoelectric points and carbohydrate content.[23] However, all are metalloproteases with a requirement for Ca^{2+} and Zn^{2+} – the latter being bound at the active site of the enzyme.[24] They operate over a neutral pH range from 5.2 to 9.6 with optimal activity at 7.5 to 8.0.[25]

Assays and substrates

The most commonly used substrate for collagenase assay is collagen itself, usually labelled with ^3H or ^{14}C and formed into fibrils. Assays using collagen have been reviewed in detail by Harris and Vater.[26] Innovations since their review include increasing the assay sensitivity by separation of collagen degradation products by SDS-PAGE and assay by fluorography;[27] decreasing the "trypsin blank" in the assay by pretreatment of the collagen with pepsin to remove the telopeptides[28] and the use of collagen films in microtitre wells allowing rapid assay of multiple samples.[29]

Some synthetic peptide substrates for collagenase have been identified. The substrate specificities determined with these compounds give direction to the design of inhibitors of collagenases.

Nagai *et al.*[30] first identified cleavage of peptides by tadpole collagenase and developed this to provide as assay for collagenase.[31] The peptides were based on the amino acid sequence at the cleavage site of whole collagen. Seven dinitrophenyl peptides were prepared of which DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2 was hydrolysed fastest but DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH was preferred as a substrate due to its greater resistance to degradation by serum peptidases. The assay involves a time consuming solvent extraction of the DNP fragments and poor solubility limits the range of substrate concentration. More recently Weingarten and Feder[32] have described the thiopeptolide Ac-Pro-Leu-Gly-"S-Leu"-Leu-Gly-OEt as a collagenase substrate with cleavage to yield "HS-Leu"-Leu-Gly-OEt which is readily determined by reaction with Ellman's reagent. This substrate has a K_m of 4 mM and K_{cat} of 370,000 h^{-1} for human skin collagenase. However, the substrate suffers from significant spontaneous hydrolysis above pH 7 and the thiol product is a collagenase inhibitor (see below). These authors also examined the action of collagenase on a series of peptides assaying the products by HPLC.[33] They demonstrated that for a substrate Leu was preferred to Ile as P_1' (nomenclature of Schechter and Berger[34]) and Leu to Ala as P_2'. They identified Ac-Pro-Leu-Gly-Leu-Leu-Gly-OEt as a good substrate for human skin fibroblast collagenase. The action of a number of collagenases was compared on analogues of this substrate in which P_1' was varied.[35] For each enzyme P_1'. Leu was optimal but the ratio of activities against alternative residues in this position varied markedly indicating that different collagenases have some differences in subsite specificity. These differences may be used to produce inhibitors which are selective/specific for certain collagenases.

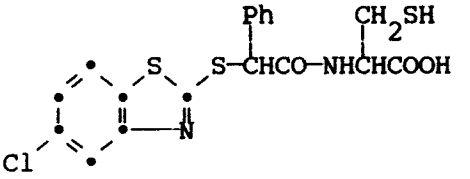
We have confirmed that Ac-Pro-Leu-Gly-Leu-Leu-Gly-OEt is a useful collagenase substrate and have used it to develop a rapid colourimetric assay for the inhibition of human synovial collagenase. The cleavage product H-Leu-Leu-Gly-OEt is estimated by reacting the newly formed terminal amino group with picrylsulphonic acid to give the trinitrophenyl peptide which is estimated spectrophotometrically at 335 nM. The assay requires partially purified enzyme but allows rapid screening of collagenase inhibitors, the classification of inhibitors and determination of inhibition constants. The K_m for this substrate with human synovial collagenase was determined as 0.57 mM at pH 7.5. I_{50} values for inhibitors we describe (see below) were determined with a substrate concentration of 0.5 mM.

Bond *et al.*[36] have recently reported a fluorimetric assay for vertebrate collagenase using dansyl-Pro-Gln-Gly-Leu-Ala-Gly-D-Arg-OH as substrate. The N- and C-terminal substituents increase its resistance to other proteases. The N-terminal cleavage product dansyl-Pro-Gln-Gly-OH is conveniently separated by TLC for semiquantitative assay or by HPLC.

INHIBITOR STUDIES ON THE MAMMALIAN COLLAGENASES

The mammalian collagenases, being zinc enzymes and having a requirement for calcium ions, are inhibited by the chelating agents EDTA and 1,10-phenanthroline at concentrations in the millimolar range.[37,38] A number of thiols have been reported to

inhibit the enzymes at similar concentrations, these include cysteine,[38] dithiothreitol[38] and cysteine or penicillamine containing peptides.[39–41] A number of naphthalene[42–44] or benzothiazole[45] containing compounds have been described, which prevent the degradation of collagen fibrils by human collagenases at concentration between 10–100 micromolar. One example of this class of compound is WY-45,368, *N*-[[(5-chloro-2-benzothiazolyl)thio]phenyl-acetyl]-L-cysteine,[46] with an I_{50} of 10 μM against human fibroblast collagenase using both Type I and Type II collagen as substrates.



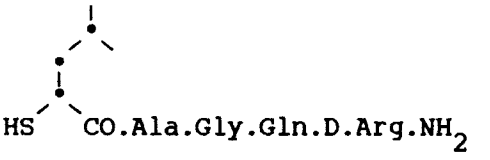
WY-45,368

WY-45,368 does not inhibit thermolysin, angiotensin converting enzyme or clostridial collagenase but it inhibits human leucocyte elastase when tested against succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide or elastin-congo red complex.[47] Tetracyclines have been shown to inhibit rat PMN collagenase in the micromolar range.[48–50]

Inhibitors based on substrate structure

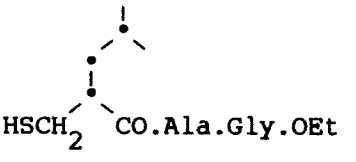
1. C-Terminal sequences containing thiol ligands

The first rational approach to the design of collagenase inhibitors was reported in 1981,[51] using an approach that had been used in the design of potent inhibitors of other zinc enzymes.[52–54] The approach is based on the assumption that the zinc ion co-ordinates to the carbonyl oxygen of the cleaved amide bond. The incorporation of a zinc ligand at the cleavage site in the substrate sequence should result in enzyme inhibition. The most active compound reported in this work was a 2-mercapto-4-methylpentanoyl tetrapeptide (I) with an I_{50} = 10 μM against tadpole backskin collagenase.



(I)

A similar approach was reported by Sundeen and Dejneka[55–57] but incorporating a methylene spacer between the ligand and the side chain to give derivatives of 2-mercaptomethyl-4-methylpentanoic acid. No biological data was reported for these compounds. The same ligand and spacer has been used to give compounds with activities below 10 μM (II).[58]

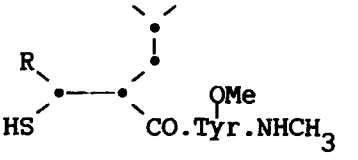


(II)

isomer A I_{50} = 1–4 μM
isomer B I_{50} = 4–10 μM

In this series of compounds both diastereoisomers possessed significant activity and increasing the length of the inhibitor did not markedly increase potency. The preference of the enzyme for a leucine side chain adjacent to the cleavage site was confirmed, replacement of the isobutyl group by phenyl or methyl significantly reduced activity.

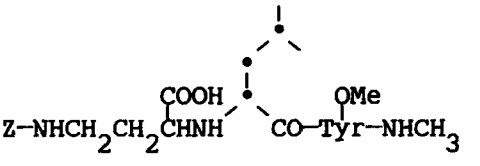
A further improvement in activity has been achieved by the alkylation of the methylene spacer adjacent to the thiol ligand.[59] In a series of acylated amino acid amides (III) the nature of the R group and the stereochemistry at both centres had a marked effect on the activity. The most active compounds (R = Me or Ph) had I_{50} values of 2.2 and 2.7×10^{-7} M.



(III)

2. C-Terminal sequences containing other ligands

The use of the substituted N-carboxymethyl ligand (IV),[60,61] first reported in potent inhibitors of angiotensin converting enzyme,[62] has resulted in activities in the micromolar range.



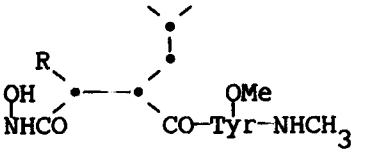
$I_{50} = 0.8\,\mu M$

(IV)

(Z = benzyloxycarbonyl)

The preferred stereochemistry for collagenase inhibitors is opposite to that required by ACE. Variation or replacement of the $Z\text{–}NHCH_2CH_2\text{–}$ portion of the molecule gives compounds with activities ranging from 1.2–91 μM[60] but changes in the tyrosine O-alkyl and the amide N-alkyl groups has little effect on the activity (0.8–3.6 μM).[61]

The most potent inhibitors of the mammalian enzymes reported to date, contain the bidentate ligand derived from hydroxylamine (V).[63] This ligand has been used successfully in inhibitors of other zinc containing enzymes.[64,65]



(V)

When R = H, the activities of the two diastereoisomers is 0.02 and 4.0 μM. In this series of compounds, the addition of an extra alkyl group ($R = CH_3\text{–}$) gives no

improvement in activity (I_{50} = 0.40, 20.0, 0.02, 0.3 μM for the four optical isomers). The requirement of the enzyme for a leucine or isoleucine residue adjacent to the cleavage site is again demonstrated with these compounds. Removal of the isobutyl group or replacement by methyl gives compounds which are at least 10^4 times less active.

Other hydroxamate containing peptides,[66] inhibit both the collagenase and proteoglycanase formed by IL-1 stimulation of chondrocytes.

3. N-Terminal sequences containing ligands

Few inhibitors based on the sequence N-terminal to the collagenase cleavage site have been reported. Starting from acetyl hydroxylamine, CH_3CONH-OH (I_{50} = 40 mM) and adding sequence related groups, resulted in a peptide hydroxamate, Z-Pro-Leu-Gly-NHOH (I_{50} = 40 μM). Corresponding peptides with different C-terminal functional groups, such as carboxylate, aldehyde and amide, showed little or no inhibition.[67] Acetyldipeptide thiolacids (R-CO-SH) inhibit the collagenase released from IL-1 stimulated chondrocytes.[66]

Systematic study of ligands and sub site-requirements

Our approach to the design of a therapeutically useful collagenase inhibitor, which commenced in late 1984, was to study three types of inhibitor based on the synthetic hexapeptide substrate Ac-Pro-Leu-Gly-Leu-Leu-Gly-OEt and the report that the zinc ion was involved with the catalytic mechanism and, therefore, in the region of the cleaved amide bond. With the ligand at the cleavage site, the peptide can be extended to:

a) the N-terminal end of the substrate (Ac-Pro-Leu-Gly-LIGAND)

b) the C-terminal end (LIGAND-Leu-Leu-Gly-OEt) or

c) in both directions (Ac-Pro-Leu-Gly-LIGAND-Leu-Leu-Gly-OEt).

It was our intention to map each subsite specificity, determine the contribution of the different side-chains and to examine a wide range of ligands.

(a) N-Terminal peptides

Using hydroxamic acid as the ligand, extension of the peptide towards the N-terminal of the substrate sequence gave improved activity (Table II). The incorporation of the P_3-proline residue gave an improvement of 50 fold, Ac-Leu-Gly-NHOH (I_{50} 500 μM) and Ac-Pro-Leu-Gly-NHOH (I_{50} 10 μM). The specificity of binding is demonstrated by the inactivity of the protected intermediate, Ac-Pro-Leu-Gly-NHOBzl, and the compounds containing an extra carbon atom, Ac-Pro-Leu-β-Ala-NHOH. Several compounds were prepared in an attempt to identify the nature of the acetylproline binding site. The proline may be replaced by alanine, with reasonable retention of activity and the acetyl group may be substituted, CF_3CO–, or replaced (CH_3SO_2–, HCO–, t-butyloxycarbonyl) without significant loss of activity. Further work is required to identify the nature of the acylproline-enzyme interaction. A thiol containing compound, Ac-Pro-Leu-$NHCH_2CH_2SH$, was significantly less active (100 μM) than the hydroxamate.

TABLE II
Inhibitors Related to the N-Terminal Side of the Cleavage Site

Structure	I_{50} (μM)
Ac-Gly-NHOH	NA
Ac-Leu-Gly-NHOH	500
Ac-Pro-Leu-Gly-NHOH	10
Ac-Pro-Leu-Gly-NHOBzl	NA
Ac-Ala-Leu-Gly-NHOH	15
TFA-Pro-Leu-Gly-NHOH	7
Boc-Pro-Leu-Gly-NHOH	22
HCO-Pro-Leu-Gly-NHOH	6
CH_3SO_2-Pro-Leu-Gly-NHOH	39
Ac-Pro-Leu-βAla-NHOH	NA

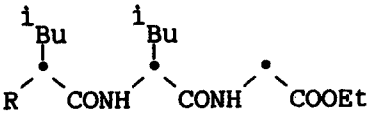
NA = not active

(b) C-Terminal peptides

(i) Ligand replacements. Replacement of the amino group in the hexapeptide substrate product, H-Leu-Leu-Gly-OEt, by zinc ligands resulted in some weak inhibitions of the enzyme (Table III). Thiol and hydroxamate (CO-NHOH) were both active (8 μM and 29 μM respectively) but formylhydroxylamine (–N(OH)CHO) and phosphonate were essentially inactive. However, insertion of a methylene spacer between the ligand and the α-centre resulted in markedly improved activity for all ligand types, the hydroxamate being the most active (0.04 μM). Incorporation of an additional methylene group or N-substitution of the hydroxamate and larger acyl groups on the hydroxylamine (–N(OH)$COCH_3$) gave less active compounds.

The situation with thiols is more complex. The compounds $HS(CH_2)_nCH(^iBu)CO$-Leu-$NHCH_3$ (n = 1 and 2) are equipotent. In agreement with the report from Spatola's group,[58] both diastereoisomers are active with this ligand system (n = 1: 3.5 and 7.0 μM; n = 2: 4.0 and 9.0 μM). This finding has been used to position the zinc

TABLE III
Effect of Ligand and spacer group in C-terminal sequence



R	I_{50} (μM)	R	I_{50} (μM)	R	I_{50} (μM)
–SH (*S*)	8.5	$-CH_2SH$ (*RS*)	1.3	–	
–CONHOH (*RS*)	29	$-CH_2CONHOH$ (*RS*)	0.04	$-(CH_2)_2CONHOH$ (*RS*)	> 10
–N(OH)CHO	⩾ 100	$-CH_2N(OH)CHO$ (*RS*)	0.3	$-CH_2N(OH)COCH_3$	60
$-PO(OH)_2$	> 100	$-CH_2PO(OH)_2$	1.3	$-CH_2CON(OH)CH_3$	15

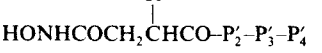
ion with respect to the specificity side chain in model building experiments. This inconsistency between thiol and hydroxamate compounds probably reflects the relative potency of the two series (10^{-6} *vs* 10^{-8} M). As the binding between the inhibitor and enzyme is improved, it is more specific and additional constraint is placed on the inhibitor structure. Alternatively hydroxamate, acting as a bidentate ligand has fewer degrees of freedom than the monodentate thiol.

(ii) Side chain replacements. The effect on inhibitor activity of changes in the four binding subsites $P_1' - P_4'$ were investigated with a view to identifying the groups essential for enzyme binding (Table IV).

(VI)

Changes in P_1': In the natural substrate this is the specificity side chain and should be limited to Leu/Ile related groups. Inhibitors containing isobutyl (Leu), secbutyl (Ile) and propyl (Nle) are all active. There is a binding contribution from both leucine methyl groups but the methyl group of isoleucine does not bind. Change of stereochemistry from *R* to *S*, removal of the side chain or its replacement by groups from

TABLE IV
Changes in subsites P1′ to P4′



R	P_2'	P_3'	P_4'	I_{50} (μM)
(*R*)-$CH_2CH(CH_3)_2$	Leu	Gly	–OEt	0.026
(*S*)-$CH_2CH(CH_3)_2$	Leu	Gly	–OEt	> 1
(*RS*)-$CH(CH_3)C_2H_5$	Leu	Gly	–OEt	0.13
(*R*)-$CH_2CH_2CH_3$	Leu	Gly	–OEt	0.07
–H	Leu	Gly	–OEt	> 100
(*R*)-CH_2Ph	Leu	Gly	–OEt	> 1
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	–NHEt	0.2
(*RS*)-$CH_2CH(CH_3)_2$	Tyr(OMe)	Gly	–NHEt	0.2
(*RS*)-$CH_2CH(CH_3)_2$	Lys(Boc)	Gly	–NHEt	0.25
(*RS*)-$CH_2CH(CH_3)_2$	Val	Gly	–NHEt	0.2
(*RS*)-$CH_2CH(CH_3)_2$	Ala	Gly	–NHEt	1.3
(*RS*)-$CH_2CH(CH_3)_2$	Pro	Gly	–NHEt	2.0
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	–OEt	0.04
(*RS*)-$CH_2CH(CH_3)_2$	Leu	L-Ala	–OEt	0.02
(*RS*)-$CH_2CH(CH_3)_2$	Leu	D-Ala	–OEt	3.0
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Pro	–OEt	0.6
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Leu	–OEt	1.5
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	$-NH_2$	0.2
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	–NHAmyl	0.25
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	$-CH_3$	0.03
(*RS*)-$CH_2CH(CH_3)_2$	Leu	Gly	–OH	0.09
(*RS*)-$CH_2CH(CH_3)_2$	Leu	$-NHCH_2CH_2-$	–OMe	0.03
(*RS*)-$CH_2CH(CH_3)_2$	Leu	$-NHCH_2CH_2-$	$-CH_2CH_2CH_3$	0.07
(*RS*)-$CH_2CH(CH_3)_2$	Leu	$-NHCH_3$		0.05

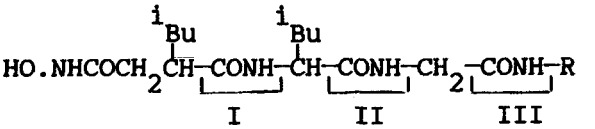
other amino acids, e.g. benzyl group (Phe), all yield totally inactive compounds. Compounds containing a hetero atom, $-X-CH(CH_3)_2$ (X = O, S or NH) are all inactive.

Changes in P_2': The requirements for this position were identified using a series of peptide ethylamides, HO-$NHCOCH_2CH(^iBu)$CO-AA-Gly-NHEt. The replacements were selected on their size and shape in an attempt to define the size of the binding site. All substituents selected, except for proline, were more active (0.2 μM) than the natural residue, alanine (1.3 μM). Binding at this position appears to come from an enzyme interaction up to the C^γ-position of the inhibitor side chain. No upper size limitation has been found at this position, for example, N^ε-protected lysine derivatives are accommodated, indicating that this side chain points towards the solvent rather than the enzyme.

Changes in P_3': In the substrate this position is always occupied by a glycine residue. Most replacement at this position are not acceptable, yielding weakly active compounds. It was unexpected to find improved activity with the L-alanine analogue. The D-alanine analogue was significantly less active. The enzyme subsites S_3' must be a small stereospecific pocket.

Changes in P_4': In a series of peptide amides $HONHCOCH_2CH(^iBu)$CO-Leu-Gly-NHR, increases in the size or shape of the R group had little effect on the activity. In collagen sequences this position is occupied by a large variety of residues. The non-specific nature of this side-chain is similar to P_2' and probably indicates that it is on the solvent side of the enzyme-bound inhibitor.

(iii) Amide bond replacements.



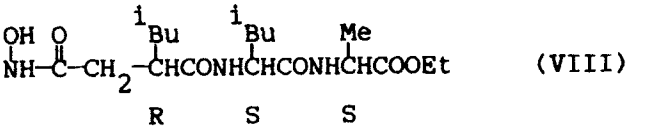
(VII)

Amide I: Replacement of amide I with an ester function (–CO–O–) or by retroamide (–NHCO–) yields inactive compounds.

Amide II: Replacement by ester gives an inactive compound. Methylation (CO–$N(CH_3)$–) reduces activity, this is consistent with the low activity of the compound containing proline in P_3'.

Amide III: Replacement of this amide by carboxylic acid (CO–OH) or ester (CO–OR), unexpectedly gave improvement in activity. Compounds containing ketone (–CO–CH_2–) or ether (–CH_2–O–) linkages indicated that the improvement in activity was due to the presence of the oxygen atom in place of –NH. Replacement with a carbon spacer (–CH_2CH_2–) gave a compound with activity between the ether and the amide. These unexpected structure-activity relationships probably reflect conformational changes induced in other parts of the molecule rather than a specific interaction between these groups and the enzyme. This interpretation is consistent with the later finding that this amide may be removed from the inhibitor without significant loss of activity.

When all the optimum groups were incorporated into one structure, the very potent collagenase inhibitor (VIII) was obtained, $I_{50} = 8.5 \times 10^{-9}$ M ($K_I = 5.0 \times 10^{-9}$ M).



(VIII)

(c) Inhibitors containing both N and C terminal substrate sequences

For this class of compound the chosen ligand was the substituted N-carboxymethyl group (Table V). The pentapeptide derivative, Ac-Pro-Leu-$NHCH_2CH(COOH)$-$NHCH(^tBu)CO$-Leu-$NHCH_3$ has an activity of 2.5×10^{-6}M but this is almost identical to the much shorter compound $CH_3CH(COOH)NHCH(^tBu)CO$-Leu-$NHCH_3$ ($I_{50} = 1\text{–}2 \times 10^{-6}$M), suggesting that the N-terminal Ac-Pro-Leu portion of the molecule does not bind to the enzyme. The peptide containing the N-terminal sequence but lacking the essential C-terminal end of the peptide, Ac-Pro-Leu-$NHCH_2CH(COOH)$-$NHCH(^tBu)COOH$ is active at 30 μM. It would appear that with this ligand either the N-terminal or the C-terminal end of the molecule binds to the enzyme but not both. These findings are all consistent with the inhibition data reported by the Searle group.[60,61]

To date, the search for other ligands closer in geometry to the amide group, which would allow both ends of the molecule to bind, has met with mixed success. Compounds containing replacements for the cleaved amide, for example $-CH(OH)CH_2-$ and $-COCH_2-$ have been very weakly active but the ketomethyleneamino compound, Z-Pro-Ala-$NHCH_2CO$-CH_2-Leu-Ala-Gly-OEt ($K_i = 60 \pm 16\,\mu$M)[68] and a series of

TABLE V

Peptides which cross the cleavage site

–Gly-Pro-Leu-Gly–Leu-Leu-Gly–	I_{50} (μM)
$CH_3CH(COOH)$–NH^tBuCHCO–Leu–NHEt	18
$CH_3CH(COOH)$–NH^tBuCHCO–Leu–NHEt	1–2
Ac-Pro-Leu-$NHCH_2CH(COOH)$–NH_2	INACTIVE
Ac-Pro-Leu-$NHCH_2$-L-$CH(COOH)$–NH^tBuCHCOOH	90
Ac-Pro-Leu-$NHCH_2$-D-$CH(COOH)$–NH^tBuCHCOOH	30
Ac-Pro-Leu-$NHCH_2CH(COOH)$–NH^tBuCHCO–Leu–NHEt (D)	2.5

phosphonamides (K_i = 14–78 μM), in which the carbonyl at the cleavage site is replaced by PO(OH), show reasonable activity.[69]

Computer models of inhibitors

Since the three-dimensional structure of mammalian collagenases has not been elucidated we have investigated the sub-site requirements of the enzymes by building computer graphics models of collagenase inhibitors using two criteria:

1. The structural requirements of the unusual substrate collagen and
2. Structural similarities in the active site topologies of zinc proteases based on the convergence of active centre geometries[70,71] of similar enzymes like thermolysin and carboxypeptidases.

1. The "Collagen" model

The backbone conformation of a repeating tripeptide polymer based on collagen[72,73] has been used as a template for the construction of leucyl-leucyl-alanine peptides (Figure 2a). It was found that the change from glycyl-prolyl-hydroxyproline to leucyl-leucyl-alanine did not require any substantial rearrangement of the polypeptide backbone in order to obtain a stereochemically feasible model. The bidentate binding

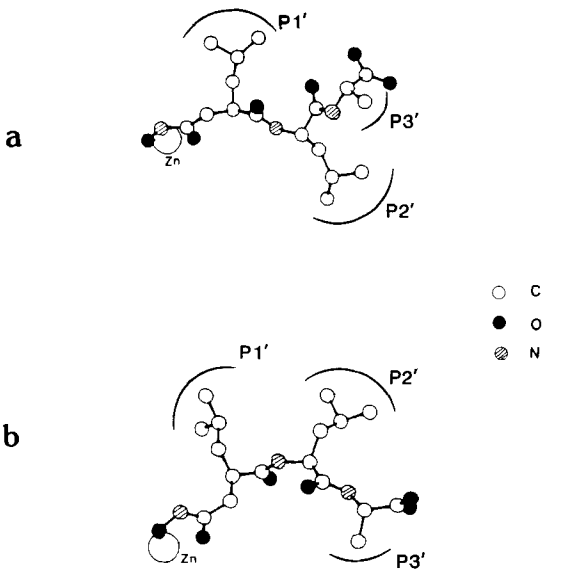


FIGURE 2 Computer graphics models of the collagenase inhibitor, based on structure VIII. (a) Model based on the predicted conformation of collagen monomers; (b) Model derived from the substitution of side chains on the thermolysin inhibitor (2-benzyl-3-mercapto-propanoyl)-L-Ala-Gly-amide. Only the non-hydrogen atoms are shown. The probable location of the catalytic zinc (in the enzyme) is indicated.

of the hydroxamate group to the catalytic zinc has been modelled on the observed data on zinc-hydroxamate interactions in small organic molecules.[74,75]

2. The "Active Site" model

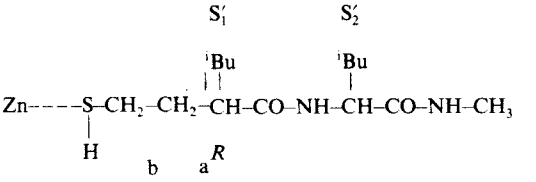
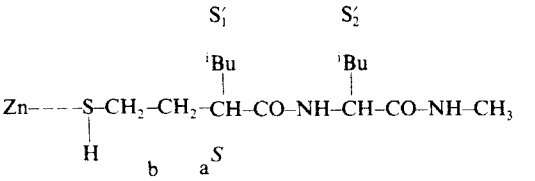
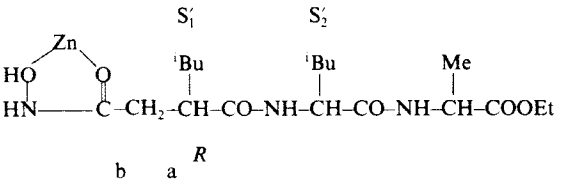
The crystal structure of the complex of (2-benzyl-3-mercapto-propanoyl)-L-Ala-Gly-amide with the zinc enzyme thermolysin has been studied[76] at 1.9 Å resolution. On the premise that there is structural homology in zinc enzymes similar to that observed for serine[70] and thiol[71] proteases, a second model for the inhibitors was obtained by the replacement of the side chains of the thermolysin inhibitor by the collagenase preferred sequence Leu-Leu-Ala (Figure 2b).

It is apparent from Figures 2a and 2b that the two models are substantially different, with the principal distinction being the disposition of the leucyl side chains (i.e. residues P_1' and P_2'): on the same side of the polypeptide backbone in the "Active Site" model and on opposite sides of the backbone in the "collagen" model. However, as reported above the SAR's of various residue replacements in the sub-site P_2' had indicated that the side chains in this position are towards solvent. These data suggested that the "collagen" (Figure 2a), which has the P_2' sub-site spanning a region on the opposite side of the specificity pocket P_1' was a more plausible working model for collagenase inhibitors.

The location of the zinc atom in relation to the specificity side chain P_1' in Figure 3 was derived by correlating a computer graphics derived mesh of the loci[77] of selected atoms around the zinc-chelation groups in the structures with their relative potency (Table VI).

TABLE VI

Chemical structures and relative potencies of inhibitors used to determine the loci of the zinc chelating group. 'a' and 'b' indicate the rotatable single bonds

		potency
i.		4.0 μM
ii.		9.0 μM
iii.		8.5 nM

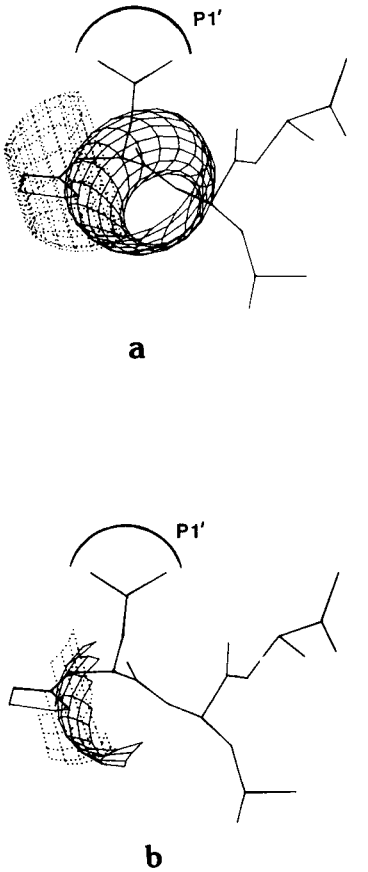


FIGURE 3 (a) Loci of the thiol group of the inhibitors (i) (dotted) and (ii) shown in Table VI. A line drawing of part of the inhibitor (iii) is shown and the specificity pocket is identified; (b) Area common (< 1Å) to the two loci shown in Figure 3a. Only non-hydrogen atoms are shown.

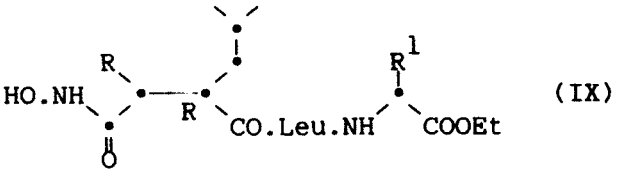
Structural models of inhibitors i, ii and iii shown in Table VI were generated using the conformation obtained from the collagen model (Figure 2a). By allowing free rotation about the single bonds a and b indicated in Table 6, the possible locations of the terminal atoms in the three structures were obtained.

Since the location of the first isobutyl side chain in compounds i and ii must be in the same specificity pocket (sub-site S_1' of the enzyme), it was possible to overlay the loci of the S-atoms in the two inhibitors by matching the relevant isobutyl groups (Figure 3a). Also, since both inhibitors have comparable potency, the catalytic zinc must be accessible to the thiol groups of both structures, so that it is reasonable to assume that the location of the zinc atom is close to the region of nearest overlap (< 1 Å, Figure 3b) of the two superimposed loci.

A similar procedure was repeated for the accessible surface area of the hydroxamate group of structure iii, Table VI, and a reduced mesh of closest overlap (largest deviation 1 Å) of all three structures was obtained. The location of the zinc atom (as shown in Figure 2) was then estimated by allowing bidentate ligand binding of the hydroxamate group at the region of maximum overlap of reduced loci.

Peptide mimetics

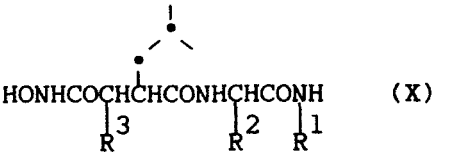
From the model (Figure 2a) it was predicted that a stereospecific substitution at the methylene group adjacent to the hydroxamate would be allowed (R in structure IX).



(IX)

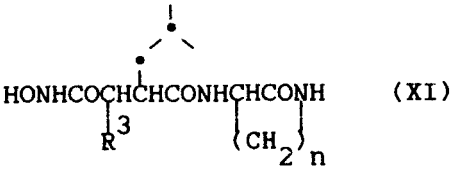
The presence of a methyl group at this position (R = CH_3–; R^1 = H) gave slightly improved activity for the *S*-isomer, the *R*-isomer was relatively inactive (R = CH_3–; R^1 = H; I_{50} = 0.02 μM, R = R^1 = H; I_{50} = 0.026 μM). The use of a larger group (R = –$(CH_2)_4$–NPth) gave further improvement in activity (R^1 = H, I_{50} = 0.012 μM); R^1 = CH_3–; I_{50} = 0.005 μM). The improvement in activity is probably due to increased conformational restraint, fixing the orientation of the ligand with respect to the specificity side chain. The increased restraint in the alkyl compounds is demonstrated by the n.m.r. spectra, which shows only one conformation in solution. The conformation is in good agreement with the model derived from the collagen structure.

From an analysis of the SAR's and the model, the inhibitors possess three groups in the molecule which point away from the enzyme (R^1 to R^3 in structure X).



(X)

These groups may be joined together in pairs to give cyclic mimetics, which should possess improved *in vivo* stability. Joining groups R^1 and R^2 gives lactams (XI).



(XI)

For n = 4, both the *S* and *R* isomers have activities in the micromolar range (*S* = 4.2 μM; R = 7.2 μM) but as the ring size is increased the activity improves. When n = 6 the I_{50} = 0.1 μM and for n = 10 the (–) isomer has an I_{50} = 0.026μM (Figure 4). These potencies parallel the amount of trans amide conformation in the different lactams.

The groups R^1 to R^3 in structure (X) may also be used to modify the overall properties of the molecule, for example, R^2 = $-(CH_2)_4NH \cdot CO \cdot C_6H_4 \cdot COONa$ or $-(CH_2)_4NH \cdot CO \cdot CH_2NH_2 \cdot HCl$, gives compounds with greatly improved water solubility without significant loss of activity. The use of the fluorescent dansyl group at this position gives compounds which are of use in the *in vivo* assessment of the pharmacokinetics of the compounds.

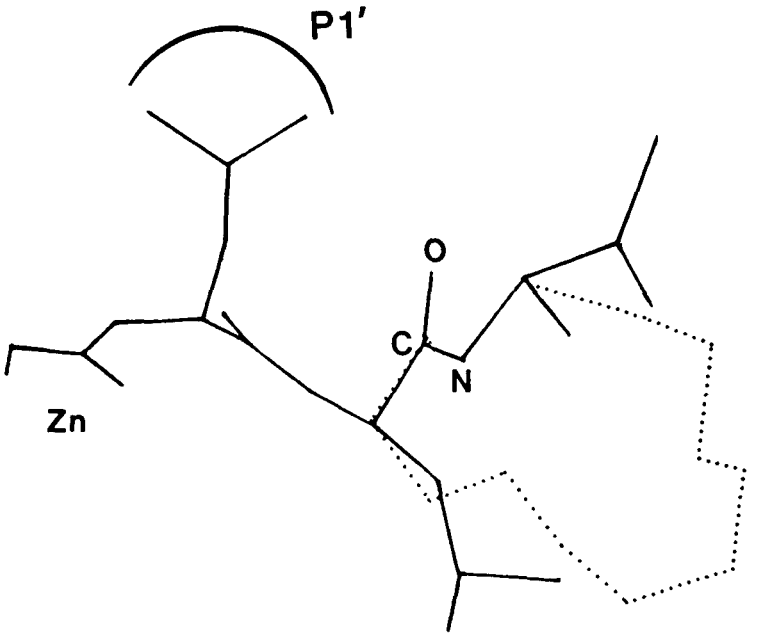


FIGURE 4 Superposition of a thirteen-membered lactam on the "collagen" model of collagenase inhibitors. The specificity pocket P_1' and the superimposed amide are identified.

Current Status of Inhibitor Design

The incorporation of zinc ligands into fragments of the hexapeptide substrate has yielded potent inhibitors of the mammalian collagenases. The most active group of compounds are the hydroxamate containing C-terminal peptide sequences (I_{50} = 5–20 nM). The minimum requirements for activity in this type of compound is a ligand optimally spaced from the specificity side chain and a trans amide bond between the P_2' – P_3' residues. Further extension of P_3' or higher sub-sites has little effect on the activity. Peptides related to the N-terminal sequence have activities in the micromolar range, as do compounds designed to bind to both sides of the cleavage site.

Although good progress has been made in the design of inhibitors, more work is required to attain the additional properties of *in vivo* stability and oral absorption required of a therapeutic agent.

POTENTIAL THERAPEUTIC ROLE OF COLLAGENASE INHIBITORS IN DISEASE

There are several quite distinct common disorders in which collagen degradation is known to occur. The relative importance of this event to the overall pathology of the disease is in each case equivocal. However, in some instances the evidence strongly suggests that collagenase does play an important rôle in the pathology of the disease and thus justifies the attempt to produce collagenase inhibitors as potential therapeutic agents. Only when inhibitors are available with adequate potency, specificity

and pharmacokinetics for realistic clinical trial can the rôle of collagenase in the various disease states be properly assessed.

Arthritis

In arthritis it is the loss of articulating surface of the joint which is the major disabling factor in the disease process. The articular cartilage consists of proteoglycan within a collagen matrix. Although loss of the proteoglycan precedes loss of the collagen matrix it is the latter event which appears to be the rate limiting, essentially irreversible step in the process. In rheumatoid arthritis the source of the collagenase(s) which cause this destruction is uncertain and possibly multiple. Also non-enzymic factors, mechanical stress and free radical reactions for example, may play a rôle in collagen degradation. In this disease the synovial cavity has a high population of polymorphonuclear leucocytes (PMN), typically 10^7/ml.[78] In contrast to other cell types PMN store pro-collagenase in their specific granules and release this extracellularly on activation. However, Wize *et al.*[79] claim that, on the basis of physical properties, the pro-collagenase in rheumatoid synovial fluid is not of PMN origin but corresponds to the enzyme derived from synovial fibroblasts. This fibroblast enzyme has been identified by immunolocalisation at sites of cartilage erosion in the rheumatoid joint.[80] In addition the chondrocytes within the cartilage are also capable of producing (pro)collagenase[16] and Bromley *et al.*[81] have provided histological evidence of a bidirectional attack on knee joint cartilage with erosions in some cases emanating from the subchondral bone, possibly implicating chondroclasts. Thus a collagenase inhibitor may be of value in rheumatoid arthritis but is it likely that a broad inhibitory specificity will be required to inhibit the enzymes from a number of different sources.

In osteoarthritis the situation may be simpler. There are few PMN's in the joint and little or no proliferation of the synovial cells. In this case therefore it is likely that the chondrocyte or chondroclast are the source of degradative enzymes. Pelletier *et al.*[82] have extracted collagenase directly from human osteoarthritic cartilage. They have shown that highest levels were found in the centre of lesions and that there was some correlation between collagenase levels and disease severity. In osteoarthritis, as in rheumatoid, proteoglycan loss precedes collagen degradation. As the proteoglycanases (stromelysins) from synovial fibroblasts and chondrocytes are metalloproteinases similar to the collagenases, it may be possible to inhibit both enzyme groups with a single inhibitor.[83]

Tumour Metastasis

Metastatis is the process in which tumour cells escape from a primary solid tumour, enter vascular channels and are transported to different organs. Metastasis is the major cause of morbidity and death for patients with solid malignant tumours, the primary tumour being frequently treatable by chemotherapy, surgery and/or radiotherapy. The metastasising tumour cell must interact with, or pass through, host matrix at many stages in the process. Basement membrane appears to play a crucial rôle in the progression of invasive tumours.[84] The collagen of basement membrane is Type IV which is resistant to the common Type I–III collagenases. Liotta *et al.*[85,86] have shown that in tissue culture and animal model systems tumour cells can produce "Type IV collagenase" and that the level of enzyme activity expressed by cells correlates with their metastatic potential. This correlation has been tentatively

extended to clinical observations. Nakatsukasa[87] has measured Type IV collagen degrading activity in liver homogenates from patients with hepatocellular carcinomas. The activity seemed higher at the periphery of the carcinoma than at the centre and activity was found to be highest in a patient with many metastatic nodules in the lung. However, this picture is perhaps too simplistic. As Vaes *et al.*[88] point out, other factors like cell mobility and co-operation by host cells, e.g. macrophages, in producing collagenolytic enzymes, and resistance of tumour cells to host defences will all play a rôle in the final metastatic potential of the cells.

Thus an inhibitor of "Type IV collagenase" may have therapeutic value in reducing the rate of tumour metastasis. Levy *et al.*[89] have shown that a polypeptide collagenase inhibitor isolated from human amniotic fluid inhibits the invasion of M5076 reticulum sarcoma cells into basement membrane *in vitro*. There is clearly a specificity difference between collagenases degrading Type IV collagen and the more common collagenase active against Types I to III. This difference may be exploitable in the design of specific inhibitors of Type IV collagenase which would not effect most normal collagen turnover.

Periodontal Disease

Peridontal disease is a chronic inflammatory condition initiated by bacteria accumulating at the gingival margin and resulting in gradual breakdown of periodontal supporting structures, particularly collagen fibres, leading finally to loss of teeth. The possible identity of the collagenase(s) responsible for this collagen (largely Type I) loss has been recently discussed in detail by Uitto.[90] He concludes that although some of the invasive bacteria can themselves produce collagenase the major source of the enzyme affecting the tissues is from host cells which have been stimulated directly or indirectly in response to the infection. Much of the collagenase in crevicular fluid derives from the PMN's which enter the gingival pockets in large numbers to combat the infection. Deeper in the tissues collagenase generated by stimulated gingival fibroblasts, macrophages and epithelial cells may all play a rôle in collagen loss. Thus broad spectrum collagenase inhibitors may have therapeutic value in this disorder. Indeed Golub *et a.*[48,49] have shown that certain tetracyclines, in addition to their antibacterial action, are modest (I_{50} 1–10 μM) inhibitors of a number of mammalian collagenases and that in preliminary clinical studies minocycline can reduce levels of collagenolytic activity in crevicular fluid. They therefore propose that the tetracyclines have collagenase inhibition as an additional mechanism of action in this disorder.

Corneal Ulceration

The cornea is formed from perfectly regular orthogonal layers of collagen fibres creating a quasi-crystalline transparent structure. This is covered by an epithelium. A number of conditions including alkali burns, radiation and vitamin E deficiency can cause injury to the surface epithelium resulting in destruction of the underlying collagen, loss of structure and ulceration. The loss of this almost pure collagen tissue is most probably mediated by collagenase from either the corneal epithelium and/or corneal cells.[91] There has already been some success in the treatment of this condition by topical application of weak non-specific collagenase inhibitors such as EDTA and cysteine.[91] More recently oral tetracycline has been successfully used in a pilot study involving 18 patients with persistent corneal epithelial defects.[92]

Other Disorders

Excessive bone collagen turnover and loss appears to be involved in Paget's disease, osteoporosis, hyperparathyroidism and cholesteatoma. Excessive skin collagen breakdown appears to be involved in recessive dystrophic epidermolysis bullosa. The rôle of collagenase in these disorders, as with others cited above, will best be determined when suitable potent and specific collagenase inhibitors are available for clinical trial.

It would appear that there is wide potential clinical application for suitable collagenase inhibitors. However, as with other attempts to use inhibition of an enzyme as a mode of drug action, the normal physiological function of the target enzyme may be impaired. In the context of slow-turnover connective tissue long term toxicity will need to be vigilantly assessed.

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