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Short Communication

PHTHALOYL-GLYCYL^P-ISOLEUCYL-TRYPTOPHAN BENZYLAMIDE IS A POTENT INHIBITOR OF HUMAN SKIN FIBROBLAST COLLAGENASE WITH A K_i OF 25 nM

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INTRODUCTION

The vertebrate collagenases are zinc metalloproteases which cleave triple helical native collagen at a single Gly-Leu or Gly-Ile peptide bond about one quarter of the distance from the carboxy-terminus of each polypeptide chain[1]. Human skin fibroblast collagenase[2] appears to be immunologically and catalytically identical to human synovial collagenase[3–5]. Human neutrophil collagenase is immunologically and catalytically different[5,6]. The neutrophil enzyme may be involved in at least the initial phase of rheumatoid arthritis while the synovial enzyme is thought to be involved in the later invasive phase[7,8].

Inhibitors of synovial collagenase containing carboxyalkyl, thiol, hydroxamate, and phosphonic acid functional groups have been reviewed[9]. The best of these was a hydroxamate analog of a tripeptide with a K_i of 5 nM. A thiol inhibitor of pig synovial collagenase with an IC_{50} of 40 nM has been reported[10]. A phosphonamidate analog of a tetrapeptide inhibits human neutrophil collagenase with a K_i of 14 uM[11].

We report here phthaloyl-Gly^P-Ile-Trp-NHBzl (see Figure 1b), which inhibits pure human skin fibroblast collagenase with a K_i of 25 nM using a thiol ester substrate at pH 6.5[12]. The superscript P indicates that the carboxyl group of glycine has been replaced by the phosphonic acid group[11]. This inhibitor has a K_i of 34 nM for crude human skin fibroblast collagenase containing gelatinase and a K_i of 220 nM for crude human sputum collagenase, also containing gelatinase.

MATERIALS AND METHODS

Phthaloyl-Gly^P-Ile-Trp-NHBzl (4) was prepared in five steps. N-Bromomethylp-

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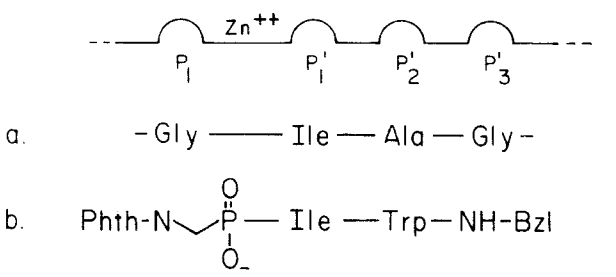

FIGURE 1 The proposed binding modes of collagen (a), and phthaloyl-Gly^P-Ile-Trp-NHBzl (4) (b) to vertebrate collagenase. The cleavage site in the collagen chain in (a) is at the Gly-Ile peptide bond[9].

hthalimide was converted in 72% yield to dibenzyl-(phthalimidomethyl) phosphonate (1) by reaction with the sodium salt of dibenzylphosphite at -15° in dimethylformamide. (1) was converted to its corresponding phosphonochloridate with PCl_5 and treated with L-isoleucine *p*-nitrophenyl ester hydrobromide to yield *N*-[(phthalimidomethyl) benzyloxyphosphinyl]-L-isoleucine *p*-nitrophenyl ester (2) in 63% yield. Compound (2) was coupled with L-tryptophan benzylamide in dimethylformamide at O°C to give *N*-[(phthalimidomethyl) benzyloxyphosphinyl]-L-isoleucyl-L-tryptophan benzylamide (3) in 87% yield. Hydrogenolysis of (3) on Pd/C methanol in an open flask, with the addition of 1 equivalent of 0.2 N $NaHCO_3$ over a period of 1 h, gave the sodium salt of phthaloyl-Gly^P-Ile-Trp-NHBzl (4) in 94% yield. The synthetic details will be given in a later publication.

Purified human skin fibroblast procollagenase was a gift from Dr. John Jeffrey, Division of Dermatology, Washington University School of Medicine, St. Louis, MO. Crude human skin fibroblast procollagenase containing gelatinase was obtained from serum free medium collected from human skin fibroblasts[2] (American Type Culture 1471) by precipitation with 55% ammonium sulfate. Electrophoresis of active crude fibroblast collagenase on a gelatin-polyacrylamide gel[13] showed a band comigrating with pure activated collagenase and a slower band thought to be gelatinase. This crude enzyme digested gelatin more rapidly than native collagen using a radioassay[14]. Crude collagenase containing gelatinase was obtained from purulent sputum (a gift from Dr. Paul Huebner, Elastin Products, Pacific MO) by homogenization in distilled water and then extraction with sodium chloride as described for purification of elastase from sputum[15]. The distilled water extract was activated with trypsin[2] and precipitated with 60% ammonium sulfate. Electrophoresis on gelatin-polyacrylamide showed bands consistent with neutrophil collagenase [70 kD[16]], and slower bands which could be gelatinase[17].

Collagenase inhibitors were assayed against 1–2 nM collagenase using the spectrophotometric assay with the thiol ester substrate Ac-Pro-Leu-Gly-SCH(iBu) CO-Leu-Leu-GlyOEt[12] at pH 6.5 in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer 10 mM in calcium chloride at 25°C in the presence of 1 mM 4,4′-dithiodipyridine. Procollagenase was activated to collagenase as described[2]. Substrate concentrations were from 100 μM to 700 μM for determinations of K_i, and 100 μM for determinations of IC_{50}. K_m was found to be 3.4 $\pm$ 0.4 mM and k_{cat} 100 $\pm$ 20 s^{-1} [K_m 3.9 mM and k_{cat} 103 per second[12]]. The velocity of the enzyme catalyzed reaction was always corrected for the spontaneous hydrolysis of thiolester substrate. Initial velocities were linear, and were recorded in duplicate and averaged. Reported K_i values

are the result of averaging K_i's calculated from Lineweaver-Burk and Dixon plots from two independent experiments or of averaging K_i's calculated from IC_{50}'s and Dixon plots. K_i and IC_{50} values for the inhibitor agreed within experimental error as expected for the low substrate concentrations employed[18]. A hexapeptide substrate (the amide analog of the thiol ester)[12] was also used to assay (4) against pure fibroblast collagenase at pH 7.5 using fluorescamine to detect hydrolysis[9,19].

RESULTS AND DISCUSSION

Figure 1a shows the vertebrate collagenase cleavage site between glycine and isoleucine (or leucine) in native collagen[9]. Figure 1b shows phthaloyl-Gly^P-Ile-Trp-NHBzl (4). Its K_i value against pure human skin fibroblast collagenase is 25 ± 5 nM when assayed against the thiol ester substrate at pH 6.5[12]. Figure 2 shows Lineweaver-Burk and Dixon plots of the inhibition of human skin fibroblast collagenase by (4). This compound was found to inhibit crude human skin fibroblast collagense containing gelatinase with a K_i value of 34 ± 7 nM. It inhibited crude collagenase from human sputum, presumably neutrophil collagenase and gelatinase, with a K_i value of 220 ± 80 nM. This inhibitor is thus one of the most potent known for fibroblast collagenase and by far the most potent phosphorus-containing transition state analog inhibitor yet reported for any vertebrate collagenases[11]. The inhibition of the crude collagenases suggests that this compound also inhibits gelatinase.

The thiol ester assay employed here has not been used before to evaluate inhibitors. Cleavage generates a mercaptan which presumably inhibits collagenase. Since this mercaptan reacts with an excess of 4-4′-dithiodipyridine in the assay to generate the 4-thiopyridine anion chromophore, it must not accumulate to a concentration sufficient to inhibit the enzyme. This thiol ester is not specific for collagenase. Gelatinase

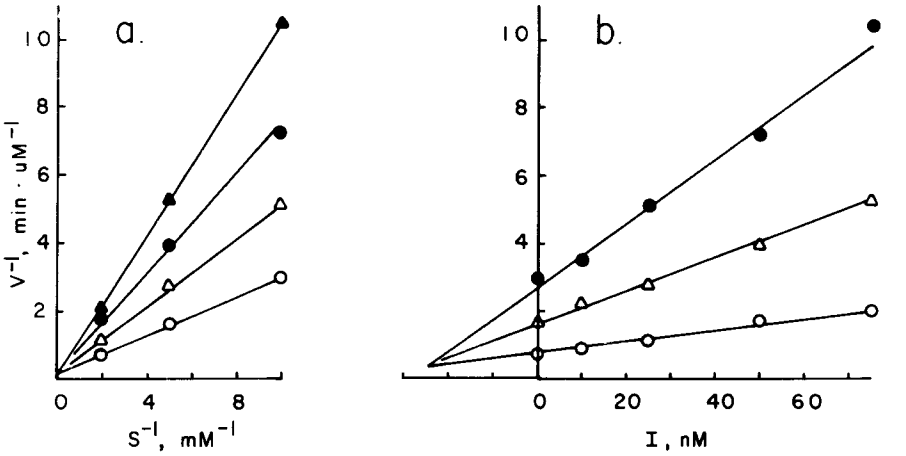

FIGURE 2 Lineweaver-Burk (a) and Dixon (b) plots of the inhibition of human skin fibroblast collagenase by phthaloyl-Gly^P-Ile-Trp-NHBzl using the thiol ester substrate at pH 6.5[12]. Inhibitor concentrations in (a) are O (○), 10 (△), 25 (●) and 75 (▲) nM. Substrate concentrations in (b) are 500 (○), 200 (△), and 100 (●) uM.

and elastase have significant activities against this substrate[19]. Finally, the assay is most conveniently performed at pH 6.5 since the spontaneous hydrolysis rate of the thiol ester is large at pH 7.5[19]. Compound (4) has a K_i value of 66 ± 2 nM when assayed against pure fibroblast collagenase using the hexapeptide analog[12] of the thiol ester at pH 7.5. We have shown that the difference in K_i values in the two assays is due only to the difference in pH.

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