INHIBITION OF PROLYL HYDROXYLASE ACTIVITY AND COLLAGEN BIOSYNTHESIS BY FIBROSTATIN C, A NOVEL INHIBITOR PRODUCED BY *STREPTOMYCES CATENULAE* SUBSP. *GRISEOSPORA* NO. 23924

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Fibrostatin C, a novel prolyl hydroxylase inhibitor produced by *Streptomyces catenulae* subsp. *griseospora* No. 23924, inhibited the activity of purified chick embryo prolyl hydroxylase by about 50% at a concentration of 2.9×10^{-5} M. The inhibition was mixed type with respect to $(Pro\text{-}Pro\text{-}Gly)_5$ with a *Ki* of 2.1×10^{-5} M. When an excess of ferrous ions or ascorbate was added to the reaction mixture, the inhibition was negligibly or slightly reversed, respectively.

Fibrostatin C, when administered intraperitoneally at 1 mg/kg/day or orally at about 100 mg/kg/day as a dietary admixture, significantly inhibited estradiol-17β stimulated collagen biosynthesis in the uterus of the immature rat.

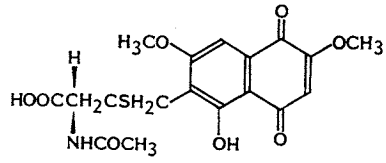
Collagen, the major structural component of connective tissue, is responsible for the functional integrity of tissues such as bone, skin, cartilage and tendon. During the biosynthesis of collagen, the conversion of prolyl residues to hydroxyprolyl residues is said to be a rate-limiting step. The hydroxyproline moiety in collagen molecules plays a critical role in the stability of the collagen triple helix[1,2]. Collagen deficient in hydroxyproline can not form a tight triple helical structure at body temperature and is easily degraded by nonspecific proteases[3].

Enhanced prolyl hydroxylase activity (prolyl-glycyl-peptide, 2-oxoglutarate dioxygenase, EC 1.14.11.2) has been detected in various experimental and pathological fibroses[4~9]. Therefore, compounds capable of selectively inhibiting prolyl hydroxylase might have clinical applications in treating fibrotic diseases.

In previous papers[10,11], it was reported that P-1894B, a potent prolyl hydroxylase inhibitor produced by *Streptomyces albogriseolus* subsp. No. 1894, significantly inhibits collagen biosynthesis when administered intraperitoneally; it is less effective when administered orally.

Fibrostatins A, B, C, D, E and F are novel prolyl hydroxylase inhibitors isolated from the culture broth of *Streptomyces catenulae* subsp. No. 23924[12,13]. Fibrostatin C (Fig. 1) is a major component in the culture filtrate and proved to inhibit effectively collagen biosynthesis when administered orally. The present paper describes the inhibition of prolyl hydroxylase activity and collagen synthesis by fibrostatin C *in vitro* and *in vivo*.

Fig. 1. Structure of fibrostatin C.



Materials and Methods

Materials

Fibrostatin C was isolated from the culture broth of *S. catenulae* subsp. *griseospora* No. 23924, IFO 12848, by the method developed by OHTA *et al.*[12]. Prolyl hydroxylase was purified from chick embryo extracts as described by TUDERMAN *et al.*[14]. The enzyme appeared entirely pure when examined by polyacrylamide gel electrophoresis[15]. (Pro-Pro-Gly)$_5 \cdot 4H_2O$ was purchased from the Peptide Institude, Inc., Osaka, Japan. Collagenase (Sigma, Type III) was purified by gel filtration on Sephadex G-200[16]. [1-^{14}C]-α-Ketoglutaric acid (sodium salt, 12 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA. All other chemicals were of the best commercially available quality.

Assay of Prolyl Hydroxylase Activity

Enzyme activity was measured by the [^{14}C]CO_2 release assay of RHOADS *et al.*[17]. The standard reaction mixture for the [^{14}C]CO_2 release assay (total volume 1.5 ml), contained 0.1 μmol sodium [1-^{14}C]-α-ketoglutarate (0.016 μCi), 1.5 μmol ascorbic acid, 0.1 μmol ferrous ammonium sulfate, 4 mg heat-denatured bovine serum albumin (Sigma Chemical Co., St. Louis, MO.), 0.1 mg bovine liver catalase (Boehringer Mannheim), 0.45 mg (Pro-Pro-Gly)$_5 \cdot 4H_2O$, 2 μg enzyme, and 50 μmol Tris-HCl buffer (pH 7.8).

To determine the influence of ferrous ion or ascorbate, the assay was repeated in the presence of excess ferrous ions, or excess ascorbate, respectively.

Reversibility of Inhibition of Prolyl Hydroxylase Activity by Fibrostatin C

Prolyl hydroxylase (2 μg) was preincubated with 100 μM fibrostatin C over ice water for 1.5 hours. The solution was then either dialysed against 0.01 M Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl and 0.2 M glycine, or subjected to gel filtration on Sephadex G-25. Recovery was determined by measuring the activity of prolyl hydroxylase in the dialysate or the eluate.

Determination of Inhibitory Activity against Collagen Synthesis *In Vivo*

The effect of fibrostatin C on the biosynthesis of collagen *in vivo* was measured by a modification of the method described by SALVADOR *et al.*[18], as follows. Estradiol-17β was dissolved in physiological saline containing 5% ethanol, and fibrostatin C was suspended in 0.2% gum arabic-saline.

Method A (Intraperitoneal Administration): Nineteen-day-old Sprague-Dawley female rats were distributed into four groups (15 rats/group). The first and second groups received fibrostatin C intraperitoneally at 1 and 10 mg/kg once daily for 6 consecutive days. The third and fourth groups received saline for 6 consecutive days. On the last 3 days, estradiol-17β (5 μg/rat) was administered to the first, second, and third groups 1 hour after fibrostatin C and the fourth group received 5% ethanol-saline. The rats were killed 24 hours after the last administration. The uteri were excised, washed with 3 ml of 70% ethanol and 3 ml of ether, and dried at 80°C for 2 hours. The hydroxyproline contents of the uteri were measured after hydrolysis in 6 N HCl (110°C, 22 hours), by the method of BLUMENKRANTZ and ASBOE-HANSEN[19]. The collagen content was calculated by multiplying the hydroxyproline content by 7.23[20]. The protein contents of the hydrolysates were measured by the ninhydrin method using L-leucine as a standard. Noncollagenous protein was calculated as the difference between total protein and collagen contents.

Method B (Oral Administration): The procedure was the same as in Method A except that the rats in groups 1 and 2 received fibrostatin C orally as a food mixture (0.035 and 0.07%).

Method C: This was the same as Method B except that collagen was analyzed by the collagenase digestion method described by PETERKOFSKY and DIEGELMAN[16], as follows. Each uterus was washed with water and homogenized in 3 ml of 0.05 M Tris-HCl buffer (pH 7.6) using a Polytron. TCA was added to the homogenate to a final concentration of 5%. The suspension was kept at 0°C for 30 minutes and the resultant precipitate was collected by centrifugation. The precipitate was resuspended in 2 ml of 5% TCA and the suspension centrifuged. This washing procedure was repeated twice. The precipitate was washed twice with ethanol - ether (3 : 1), once with absolute ether, and then dried slowly. The dried powder was dissolved in 0.1 N NaOH at a concentration of 5 mg/ml by warming at 37°C

with occasional shaking to give the substrate solution. The substrate solution (0.2 ml) was incubated with 60 μmol *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.2), 1.25 μmol *N*-ethylmaleimide, 0.25 μmol $CaCl_2$, and 20 μg (about 14 U) collagenase in a total volume of 0.6 ml was incubated at 37°C for 4 hours with shaking. The reaction was stopped by adding 0.1 ml of 35% TCA containing 0.5% tannic acid. The mixture was kept at 0°C for 30 minutes and then centrifuged. The supernatant was removed and the precipitate was washed with 0.5 ml of 5% TCA - 0.25% tannic acid. The suspension was centrifuged and the supernatant removed and added to the first supernatant. The combined supernatant fractions were hydrolyzed with 6 N HCl at 110°C for 22 hours. Proline and hydroxyproline were measured by the method of CHINARD[21], and BLUMENKRANTZ and ASBOE-HANSEN[19], respectively, and their molar ratio was calculated. The collagenase used in method C was shown to be free from nonspecific protease by its failure to degrade [^{14}C]tryptophan-labeled chick embryo protein[16].

Statistics

The data were statistically evaluated by Student's t-test.

Results and Discussion

Effect of Fibrostatin C on Prolyl Hydroxylase Activity *In Vitro*

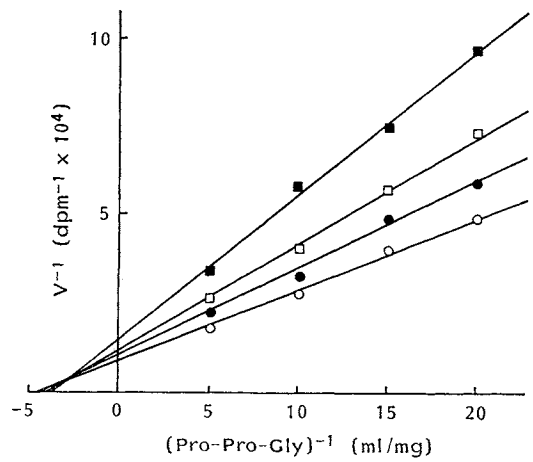
The inhibition of prolyl hydroxylase activity was found to increase with the concentration of fibrostatin C, giving a sigmoid curve; the concentration required for 50% inhibition was 2.9×10^{-5} M. Lineweaver-Burk plots revealed a mixed type of inhibition with respect to $(Pro\text{-}Pro\text{-}Gly)_5$ with a *Ki* value of 2.1×10^{-5} M (Fig. 2).

An excess of ferrous ions had little effect on the inhibition of enzyme activity by fibrostatin C (Fig. 3A). This finding indicates that fibrostatin C does not inhibit the enzyme activity by chelating ferrous ions. The results of a similar experiment conducted with an excess of ascorbate are shown in Fig. 3B. An excess of ascorbate partially protected the enzyme activity, but inhibition by fibrostatin C was not completely reversed even when ascorbate was added to the reaction mixture at a concentration about 50-fold that of fibrostatin C (Fig. 3B). This shows that fibrostatin C does not inhibit enzyme activity by acting as an antioxidant or an antagonist toward ascorbate.

MYLLYLA *et al.*[22] suggested that the superoxide anion was involved in prolyl hydroxylase reactions. Nitroblue tetrazolium, which is capable of scavenging superoxide, inhibits prolyl hydroxylase activity[22~24]. Fibrostatin C did not scavenge superoxide anion in a superoxide anion detecting system using hypoxanthine - xanthine oxidase and neotetrazolium (K. MIYATA; personal communication).

Fig. 2. Double reciprocal plots of initial rates of prolyl hydroxylase against $(Pro\text{-}Pro\text{-}Gly)_5$ concentration in the presence or absence of fibrostatin C.

○ None, ● 1.25×10^{-5} M, □ 2.5×10^{-5} M, ■ 5.0×10^{-5} M fibrostatin C.

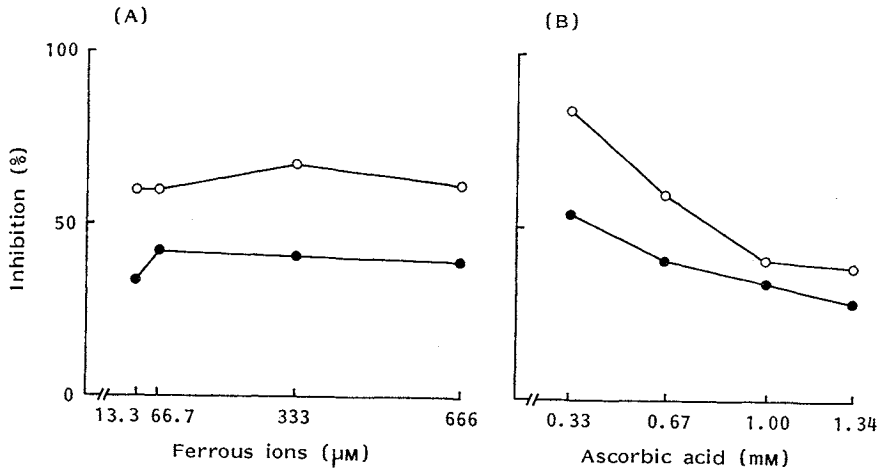


The reaction mixture and conditions are described in Materials and Methods. Each plots is the mean value of triplicate assays.

$Km=1.77\times10^{-4}$ M, $Ki=2.07\times10^{-5}$ M

Fig. 3. Effects of an excess of ferrous ions (A) or ascorbic acid (B) on the inhibition of prolyl hydroxylase by fibrostatin C.

● 25 μM fibrostatin C, ○ 50 μM fibrostatin C.



Percent inhibition was calculated with respect to the control assay mixture containing the same amount of cofactor and no inhibitor.

Each spot is the mean value of triplicate assays.

When prolyl hydroxylase was preincubated with fibrostatin C about 90% of the enzyme activity was recovered after dialysis or gel filtration. In addition to these findings, the observation that the preincubation of the enzyme with fibrostatin C before the substrate was added did not increase inhibition suggests that fibrostatin C is not a tight-binding inhibitor of prolyl hydroxylase (unpublished data).

Effect of Fibrostatin C on Collagen Biosynthesis *In Vivo*

The effect of fibrostatin C on collagen biosynthesis *in vivo* was examined using the uteri of immature rats treated with estradiol-17β. As shown in Table 1, when fibrostatin C (1 and 10 mg/kg) was injected for 6 consecutive days (Method A), the body weight of the rats was not significantly lower than that of control animals. Stimulation with estradiol-17β approximately doubled both the collagen and noncollagenous protein contents of the uterus. Fibrostatin C at 1 and 10 mg/kg significantly reduced the collagen content of the rat uterus. The noncollagenous protein content was also reduced at both dosages, but the inhibition was more specific against collagen synthesis than against noncollagenous protein synthesis (Table 1). Autopsy of the rats did not show any obviously toxic effects caused by administration of the compound for 6 consecutive days.

Total collagen content of the uterus was also significantly decreased by oral administration of fibrostatin C (food containing 0.07% fibrostatin C, Method B).

Body and uterine weights, and the noncollagenous protein content of uterus were not significantly changed (Table 2).

It is important to clarify whether underhydroxylated collagen accumulates as a consequence of inhibiting prolyl hydroxylase activity. Therefore we measured both proline and hydroxyproline in collagenase digests of uterus from fibrostatin C-treated rats (Method C). As shown in Table 3, the hydroxyproline content of the collagenase digests of uteri was reduced by fibrostatin C administered orally, but the molar ratio of hydroxyproline/proline was not changed. Collagen synthesis stimulated

Table 1. Effect of fibrostatin C on the estradiol-17β stimulated increase of collagen and noncollagenous protein (NCP) content in the uterus.

Treatment	Gain in body weight (g)	Dry weight of uterus (mg)	Total protein in uterus	
			Collagen (mg)	NCP (mg)
None	34±2	13.1±3.3	2.25±0.20	9.48±2.53
Estradiol-17β	34±2	23.2±2.5[d]	3.62±0.73[d]	17.33±3.28[d]
Estradiol-17β and fibrostatin C				
1 mg/kg	33±3	21.3±2.3	2.78±0.39[a]	15.01±1.51[a]
10 mg/kg	32±4	20.5±2.5[b]	2.60±0.36[c]	14.10±2.01[b]

Nineteen-day-old rats were given a daily injection of fibrostatin C (1 or 10 mg/kg) for 6 consecutive days as described in Materials and Mathods (Method A). The uterus was removed from each rat 24 hours after the last injection. Each value is the mean±SD, $n=15$.

[a] Significantly different from the estradiol-17β treated control at $P<0.05$.

[b] Significantly different from the estradiol-17β treated control at $P<0.01$.

[c] Significantly different from the estradiol-17β treated control at $P<0.001$.

[d] Significantly different from the 5% ethanol - saline control (none) at $P<0.001$.

Table 2. Effect of fibrostatin C on the increase of collagen and noncollagenous protein (NCP) content in the estradiol-17β stimulated uterus.

Treatment		Body weight (g)		Total protein (mg) in uterus	
		Initial	Gain	Collagen	NCP
Expt 1	Control-1	40±3	29±2	1.57±0.12	7.28±1.00
	Control-2	42±3	29±1	2.86±0.36[c]	16.72±1.93[c]
	Sample-1	40±3	28±2	2.85±0.31	15.09±2.15
	Sample-2	40±4	26±4[b]	2.38±0.34[b]	15.12±2.02
Expt 2	Control-1	39±4	32±2	1.91±0.03	7.12±0.07
	Control-2	39±3	30±3	3.51±0.40[c]	14.31±1.60[c]
	Sample-3	38±2	31±2	3.13±0.36[a]	14.15±2.29

Nineteen-day-old rats were fed for 7 days with food containing fibrostatin C (sample-1; 0.035%, sample-2 and -3; 0.07%) as described in Materials and Methods (Method A). Control groups received only 5% ethanol - saline (Control-1) or estradiol-17β (Control-2). Each value is the mean±SD, $n=15$.

[a] Significantly different from Control-2 at $P<0.05$.

[b] Significantly different from Control-2 at $P<0.01$.

[c] Significantly different from Control-1 at $P<0.001$.

Table 3. Effect of fibrostatin C on the collagenase sensitive protein of the uterus.

Treatment	Total hydroxyproline in uterus (μg)	Molar ratio (Hyp/Pro)
Control-1 (5% ethanol - saline)	149±17	0.565±0.020
Control-2 (estradiol-17β)	272±16	0.530±0.050
Estradiol-17β and fibrostatin C (0.07%)	239±26[a]	0.529±0.021

Nineteen-day-old rats were treated using the procedure described in Materials and Methods (Method C). Hydroxyproline and proline contents were measured after the digestion of uterine collagen by collagenase. Each value is the mean±SD, $n=15$.

[a] Significantly different from the estradiol-17β treated control at $P<0.01$.

by estradiol-17β was 27% less in the uterus of rats treated with fibrostatin C compared with the control group which received estradiol-17β alone. If it is assumed that the amount of newly synthesized hydroxyproline decreased by 27%, and that the resulting underhydroxylated collagen accumulated in the uterus, the molar ratio of hydroxyproline/proline should be 0.49. The actual ratio was slightly higher than this, indicating that there was no accumulation of underhydroxylated collagen. Also, underhydroxylated collagen is known to be easily degraded by tissue proteases[3].

These results indicate that orally administered fibrostatin C inhibited the hydroxylation of prolyl residues in newly synthesized collagen, and thus selectively reduced the formation of mature collagen.

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