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INHIBITION OF PROLYL HYDROXYLASE ACTIVITY AND COLLAGEN BIOSYNTHESIS BY THE ANTHRAQUINONE GLYCOSIDE, P-1894B, AN INHIBITOR PRODUCED BY *STREPTOMYCES ALBOGRISEOLUS*

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Abstract—P-1894B, a potent prolyl hydroxylase inhibitor produced by *Streptomyces albogriseolus* subsp. No. 1894, inhibited about 50% of the activity of purified chick embryo prolyl hydroxylase at a concentration of 2.2×10^{-6} M. The inhibition was noncompetitive with respect to $(Pro\text{-}Pro\text{-}Gly)_5$ with a K_i of 1.8×10^{-6} M. When excess amounts of ferrous ions or ascorbate were added to the reaction mixture, the inhibition was slightly reversed. P-1894B at a dose of 0.15 mg/kg reduced the hydroxylation of peptidyl proline and caused a significant inhibition of collagen biosynthesis in the uterus of the immature rat stimulated by the administration of estradiol-17β.

Prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate dioxygenase, EC 1.14.11.2) catalyzes the synthesis of 4-hydroxyproline in collagen. Changes in the activity of this enzyme have been studied in various experimental and pathological fibroses [1–6]. Increases in the enzyme activity accompanied by an enhanced rate of collagen synthesis have been found in various tissues. Reduction in the hydroxyproline content of collagen results in decreased thermal stability [7, 8] and increased susceptibility to tissue proteases [9]. Therefore, a compound, capable of selective inhibition of prolyl hydroxylase, might have clinical application in the treatment of fibrotic diseases.

P-1894B (Fig. 1) is a potent prolyl hydroxylase inhibitor isolated from the culture broth of *Streptomyces albogriseolus* subsp. No. 1894; its structure was determined by X-ray crystallographic analysis [10].

The present paper describes the inhibition of prolyl hydroxylase activity and collagen synthesis by P-1894B *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

P-1894B was isolated and purified from the culture broth of *S. albogriseolus* subsp. No. 1894, IFO 13881 by a method developed by Okazaki *et al.*† Prolyl hydroxylase was purified from chick embryo extracts as described by Tuderman *et al.* [11]. The enzyme was entirely pure when examined by polyacrylamide gel electrophoresis [12]. $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ was purchased from the Protein Research Foundation, Osaka, Japan. Collagenase (Sigma, Type III) was purified by gel filtration on Sephadex G-200 [13]. [1-^{14}C]Sodium α-ketoglutarate (12 mCi/mmole), L-[U-^{14}C]proline (285 mCi/mmole), L-[3,4-^{3}H]proline (41.1 Ci/mmole) and L-[2,3-^{3}H]tryptophane (11 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA. All other chemicals were of the best quality commercially available.

Assay of prolyl hydroxylase activity

The enzyme activity was measured by the $[^{14}C]CO_2$ release assay of Rhoads *et al.* [14] or the $[^{3}H]H_2O$ release assay of Hutton *et al.* [15]. The standard reaction mixture of $[^{14}C]CO_2$ release assay (total volume, 1.5 ml) contained 0.1 μmole [1-^{14}C]sodium α-ketoglutarate (0.016 μCi), 1.5 μmoles ascorbic acid, 0.1 μmole 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 $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$, 0.1 μg enzyme, and 50 μmoles Tris–HCl buffer (pH 7.8). In the $[^{3}H]H_2O$ release assay, the standard reaction mixture (total volume, 1.0 ml) contained 2×10^5 dpm [3,4-^{3}H]proline-labeled collagen substrate, 0.1 μmole sodium α-ketoglutarate, and other compounds in the same concentration as indicated above.

Assay of inhibitory activity against collagen synthesis in vivo

The effect of P-1894B on collagen synthesis *in vivo* was measured by a modified method of Salvador *et al.* [16]. Estradiol-17β was dissolved in physiological saline containing 5% ethanol. All chemicals were administered to rats by intraperitoneal injection.

Method A. Groups of 19-day-old rats (Sprague–Dawley, female, four rats/group) were given a daily administration of estradiol-17β (5 μg/rat) for 2 consecutive days. P-1894B or saline was administered

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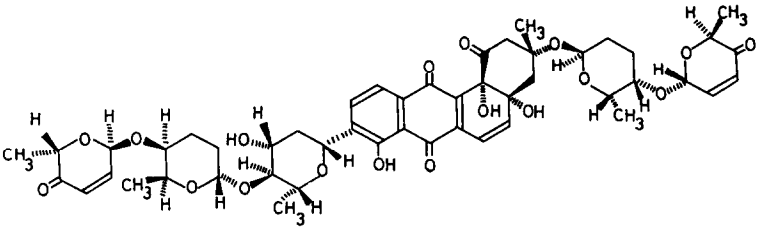

Fig. 1. Structure of P-1894B.

24 hr after the last treatment with estradiol-17β. L-[U-^{14}C]Proline (10 μCi/rat) was injected 1 hr after the treatment with P-1894B or saline. The uterus (cervix and horns) was excised from each rat 2 hr later and homogenized in 3 ml of cold 15% trichloroacetic acid (TCA). The homogenate was centrifuged at 7000 g for 10 min. The precipitate was washed with 3 ml of cold 5% TCA and extracted twice by 2 ml of 5% TCA at 90° for 60 min. Hot TCA soluble fraction (collagen fraction [17]) was hydrolyzed with 6 N HCl at 110° for 22 hr. Radioactivity of proline and hydroxyproline was measured by thin-layer chromatography in phenol–ethanol–water (3:1:1, by vol.), followed by direct scintillation spectrometry on scraped sections corresponding to proline and hydroxyproline markers, which were detected by an isatin stain.

Method B. Three-week-old rats (Sprague–Dawley, female) were divided into three groups (four rats/group). The first group received P-1894B once daily for 6 consecutive days. On the last 3 days, estradiol-17β (5 μg/rat) was administered 1 hr after the treatment with P-1894B. The second and third groups received saline instead of P-1894B for 6 consecutive days. On the last 3 days, the second group also received estradiol-17β and the third group 5% ethanol–saline. Rats were killed 24 hr after the last treatment and the uterus of each rat was excised, washed with 3 ml of 70% ethanol and ether, and dried at 80° for 2 hr. Hydroxyproline content of the dried uterus was measured in 6 N HCl hydrolysate (110°, 22 hr) by the method Blumenkrantz and Asboe-Hansen [18]. Collagen content was calculated by multiplying hydroxyproline content by 7.23 [19]. Protein content in the hydrolysate was measured by the ninhydrin method using L-leucine as standard. Noncollagenous protein was calculated as the difference between total protein and collagen contents.

Method C. In method B, the collagen of uterus was analyzed by the collagenase digestion method described by Peterkofsky and Diegelmann [13]. Each uterus was washed with water and homogenized in 3 ml of 0.05 M Tris–HCl buffer (pH 7.6) with a Polytron. TCA was added to the homogenate to give a final concentration of 5%. The suspension was kept at 0° for 30 min, and the resultant precipitate was collected by centrifugation. The precipitate was suspended in 2 ml of 5% TCA and centrifuged. This washing procedure was repeated twice. The precipitate was then washed twice with ethanol–ether (3:1, v/v), once with absolute ether, and dried slowly. The dried powder was dissolved in 0.1 N NaOH at a concentration of 10 mg/ml by warming at 37° with occasional shaking. In collagenase digestion, the reaction mixture (total volume, 0.6 ml) contained 0.2 ml of substrate solution, 60 μmoles *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffer (pH 7.2), 1.25 μmoles *N*-ethylmaleimide, 0.25 μmole $CaCl_2$ and 20 μg (about 14 units) collagenase. The mixture was incubated at 37° for 4 hr with shaking. The reaction was stopped by the addition of 0.1 ml of 35% TCA containing 0.5% tannic acid. The mixture was further kept at 0° for 30 min and centrifuged. The supernatant fraction was separated from the precipitate, and the precipitate was washed with 0.5 ml of 5% TCA–0.25% tannic acid. The resulting supernatant fraction was combined with the previous one. The combined supernatant fraction was hydrolyzed with 6 N HCl at 110° for 22 hr. Proline and hydroxyproline were measured by the method of Chinard [20] and Blumenkrantz and Asboe-Hansen [18] respectively. The collagenase used in method C was shown to be free from nonspecific proteases by its failure to degrade [^{14}C]tryptophane-labeled chick embryo protein [13].

RESULTS AND DISCUSSION

Effect of P-1894B on prolyl hydroxylase activity in vitro

The enzyme preparation was pure according to gel electrophoresis and its activity was completely dependent on ascorbate but, in the absence of added ferrous ions, about 75% of the activity of the complete assay system was left. There are conflicting reports concerning the activity of the enzyme in the absence of added iron [21, 22]; our preparation closely resembles that described by Nietfeld and Kemp [21].

The inhibition of prolyl hydroxylase activity was found to increase with the concentration of P-1894B, giving a sigmoid curve, and the concentration required for 50% inhibition was about 2.2×10^{-6} M in either assay method described in Materials and Methods (Fig. 2). Lineweaver–Burk plots revealed a noncompetitive type of inhibition with respect to (Pro-Pro-Gly)$_5$ with a K_i value of 1.8×10^{-6} M (Fig. 3). An excess of ferrous ions partially protected the enzyme activity from the inhibition by P-1894B. The inhibition by P-1894B was not reversed completely when ferrous ions were added to the reaction mixture at a concentration about 100-fold that of P-1894B (Fig. 4A). This finding indicates that P-1894B did not inhibit the enzyme activity by chelating ferrous ions. A similar experiment conducted with an excess of ascorbate is shown in Fig. 4B. An excess of ascorbate also partially protected the enzyme activity from the inhibition. This result indicates that P-1894B did

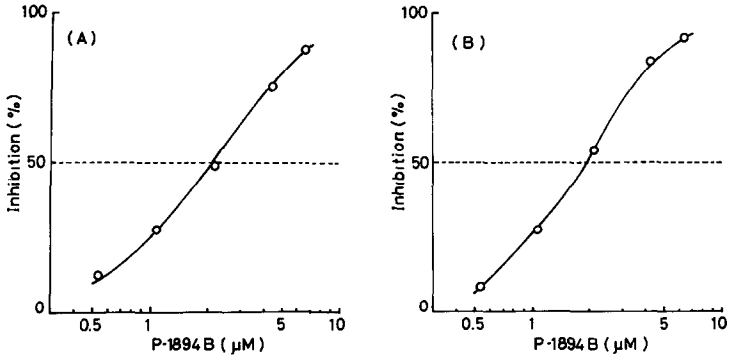

Fig. 2. Inhibition of prolyl hydroxylase activity by various concentrations of P-1894B. Assays were performed as described in Materials and Methods: (A) $[^{14}C]CO_2$ release assay, and (B) $[^{3}H]H_2O$ release assay. Results are expressed as percentages of the control (P-1894B-free). Control values were as follows: (A) complete, 4010 ± 180, minus substrate, 190 ± 5 cpm, (B) complete, 3550 ± 325, minus substrate 45 ± 3 cpm. Each spot is a mean value of triplicate assays.

not inhibit the enzyme activity by acting as an antioxidant toward ascorbate. It has been suggested that ferrous ions, ascorbate and oxygen interact at a reducing site on the enzyme, leading to the formation of superoxide anions [23, 24]. P-1894B scavenges superoxide anions in a superoxide anion generating system of hypoxanthine–xanthine oxidase using neotetrazolium as the acceptor; ferrous ions accelerate this activity of P-1894B.* This indicates that P-1894B might exert its inhibitory action by scavenging the activated oxygen intermediate, but the proof remains a problem for the future.

Prolyl hydroxylase preincubated with 10 μM P-1894B at 5° for 60 min was dialyzed against 0.01 M Tris–HCl buffer (pH 7.8) containing 0.2 M NaCl and 0.2 M glycine. About 80% of the enzyme activity was recovered. These data, and the observation that the preincubation of the enzyme and P-1894B prior to the addition of substrate did not increase the inhibition, suggest that P-1894B is not a tight-binding inhibitor of prolyl hydroxylase.

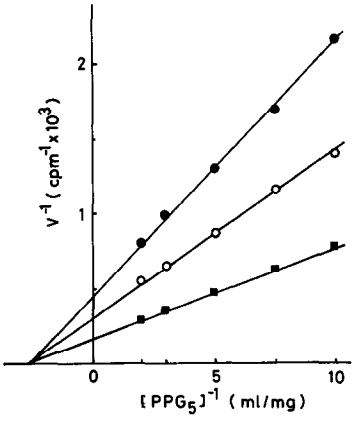

Fig. 3. Double-reciprocal plots of initial rates of prolyl hydroxylation, both in the absence and presence of P-1894B. The concentrations of P-1894B were: none (■), 1.5 μM (○) and 3.0 μM (●). The reaction was carried out under the standard conditions of the $[^{14}C]CO_2$ release assay as described in Materials and Methods, and the reaction velocity (V) was measured in cpm.

* K. Miyata (Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan), personal communication.

Table 1. Effect of P-1894B on the ratio of [^{14}C]hydroxyproline/[^{14}C]proline in uterine collagen*

Treatment		Uterine collagen	
		Total radioactivity (dpm x 10^{-4})	[^{14}C]Hyp/[^{14}C]Pro
Saline		1.73 ± 0.37	0.527 ± 0.020
P-1894B	5 mg/kg	1.66 ± 0.39	0.499 ± 0.030
	10	1.52 ± 0.29	0.522 ± 0.023
	25	1.58 ± 0.56	0.461 ± 0.012†
	50	1.71 ± 0.22	0.451 ± 0.022†

* Nineteen-day-old rats were given a daily injection of estradiol-17β (5 μg/rat) for 2 consecutive days. Either P-1894B (5–50 mg/kg) or saline was administered 24 hr after the last injection of estradiol-17β. One hour after the administration of P-1894B or saline, L-[^{14}C]proline (10 μCi/rat) was administered, and the uterus was removed from each rat 2 hr later. Each value is the mean ± S.E., N = 4.

† Significantly different from saline control at $P < 0.01$.

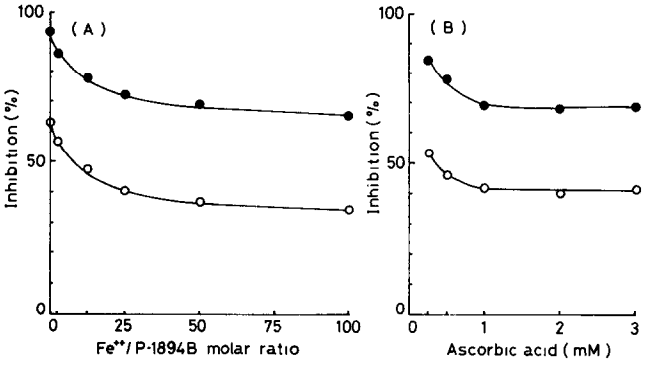

Fig. 4. Effects of an excess of ferrous ions (A) or ascorbic acid (B) on the inhibition of prolyl hydroxylase by P-1894B. Per cent inhibition was calculated with respect to the control assay mixture in the $[^{14}C]CO_2$ release assay, containing the same amount of cofactor and no inhibitor. Control values were as follows: (A) ferrous ions (in cpm), none, 2930 ± 150; 5 μM, 3730 ± 190; 10 μM, 3830 ± 190; 25 μM, 3950 ± 200; 50 μM, 3930 ± 200; 100 μM, 3930 ± 200; 200 μM, 3880 ± 195; 400 μM, 3770 ± 190; minus substrate, 200–340 cpm, (B) ascorbate, 0.25 mM, 2670 ± 130; 0.5 mM, 3370 ± 170; 1 mM, 3700 ± 180; 2 mM, 3890 ± 140; 3 mM, 3650 ± 180; minus substrate, 220–290 cpm. Each spot is a mean value of triplicate assays. The concentration of P-1894B was: 2 μM (○) or 4 μM (●).

Effect of P-1894B on collagen biosynthesis in vivo

Effects of P-1894B on *in vivo* collagen synthesis were examined using the uteri of immature rats stimulated by estradiol-17β.

As shown in Table 1, P-1894B at doses of 25 and 50 mg/kg significantly decreased the ratio of [^{14}C]hydroxyproline/[^{14}C]proline in uterine collagen. This result indicates that the P-1894B inhibited the prolyl hydroxylation reaction *in vivo*. To know whether the inhibition was specific for collagen synthesis or not, the effect of the drug on collagen and noncollagenous protein syntheses was also tested using method B. When P-1894B (0.075 to 0.300 mg/kg) was administered for 6 consecutive days, it did not cause a significant reduction in body weight. Both collagen and noncollagenous protein contents were increased about two times by estradiol-17β. Compared with the estradiol-17β-treated control, the total collagen content of the uterus was decreased significantly by P-1894B at doses of 0.15 and 0.30 mg/kg, but noncollagenous protein content was not changed significantly at any dose tested (Table 2). The difference of effective dose in the experiments of method A (Table 1) and method B (Table 2) was probably due to the solubility of P-1894B. P-1894B was scarcely soluble in water and it was administered as a suspension with gum arabic in the *in vivo* experiments. In method A, the uterus was excised 3 hr after the injection of P-1894B, which had been retained in a solid state in the injected intraperitoneal region.

Table 2. Effect of P-1894B on the increase in collagen and noncollagenous protein (NCP) content of rat uterus caused by estradiol-17β*

Treatment	Dry weight of uterus (mg)	Total protein of uterus	
		Collagen (mg)	NCP (mg)
Expt. I			
Saline	8.1 ± 1.0	1.73 ± 0.19	5.40 ± 0.84
Estradiol-17β	18.8 ± 1.5	2.54 ± 0.31	13.59 ± 1.60
Estradiol-17β + P-1894B			
0.075 mg/kg	16.3 ± 1.1	2.29 ± 0.20	11.81 ± 0.86
0.150 mg/kg	15.6 ± 1.1†	1.87 ± 0.12†	11.60 ± 0.99
Expt. II			
Saline	11.0 ± 1.8	3.12 ± 0.48	10.78 ± 1.14
Estradiol-17β	26.3 ± 6.5	4.39 ± 0.16	24.21 ± 7.21
Estradiol-17β + P-1894B			
0.150 mg/kg	24.6 ± 2.9	3.05 ± 0.39‡	21.55 ± 2.58
0.300 mg/kg	22.1 ± 3.5	3.41 ± 0.22†	26.99 ± 4.67

* Twenty-day-old rats (Expt. I) and 22-day-old rats (Expt. II) were given a daily injection of P-1894B (0.075 to 0.300 mg/kg) for 6 consecutive days; on the last 3 days, estradiol-17β (5 μg/rat) was administered 1 hr after the injection of P-1894B. Control groups received only saline or estradiol-17β. The uterus was removed from each rat 24 hr after the last injection. Each value is the mean ± S.E., N = 4.

† Significantly different from the estradiol-17β administered control at $P < 0.05$.

‡ Significantly different from the estradiol-17β administered control at $P < 0.01$.

Table 3. Effect of P-1894B (0.15 mg/kg) on the collagenase digestible protein of the rat uterus*

Treatment	Total hydroxyproline (μg)	Molar ratio Hyp/Pro
Saline	211 ± 33	0.60 ± 0.04
Estradiol-17β	366 ± 12	0.56 ± 0.03
Estradiol-17β + P-1894B 0.15 mg/kg	298 ± 5†	0.58 ± 0.02

* Nineteen-day-old rats were treated using the procedure described in the legend of Table 2. Hydroxyproline and proline contents were measured after the digestion of uterine collagen by collagenase. Each value is the mean ± S.E., N = 4.

† Significantly different from the estradiol-17β administered control at $P < 0.01$.

Murray *et al.* reported that some catechol analogues inhibited the activity of lysyl hydroxylase (EC 1.14.11.4), which catalyzes the transformation of certain lysyl residues in collagen to hydroxylysine. In this case, collagen synthesis was not inhibited and underhydroxylated collagen was accumulated in tissue culture of chick embryo calvaria [25]. To investigate this point in the present experiments, the collagenase digestion method (method C) was also carried out. The hydroxyproline content of collagenase digestion products of the uterus was decreased by P-1894B (0.15 mg/kg), but the molar ratio of hydroxyproline/proline was not changed (Table 3). It shows that newly synthesized collagen, stimulated by estradiol-17β, was decreased about 44% by P-1894B. If we assume that newly synthesized hydroxyproline was decreased 44% by P-1894B and that the underhydroxylated collagen corresponding to its inhibition was accumulated in the uterus, the molar ratio of hydroxyproline/proline would be calculated to be 0.42. Therefore, it is clear that underhydroxylated collagen was not accumulated in the uterus. Underhydroxylated collagen is known to be degraded easily by tissue proteases [9], and the peptides degraded from procollagen are also known to inhibit collagen synthesis [26, 27]. The reduction of collagen content in the uterus by P-1894B is probably due to these regulatory systems of collagen synthesis.

The above results indicate that P-1894B inhibited the hydroxylation of prolyl residues in collagen and reduced collagen synthesis more selectively than noncollagenous protein synthesis *in vivo*.

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