# Inhibition of prolyl 4-hydroxylase *in vitro* and *in vivo* by members of a novel series of phenanthrolinones

Trevor J. FRANKLIN*[1], William P. MORRIS*, Philip N. EDWARDS*, Michael S. LARGE* and Robert STEPHENSON†

*Research Department, AstraZeneca, Alderley Park, Macclesfield, Cheshire, SK10 4TG, U.K., and †FibroGen Inc, 225 Gateway Boulevard, South San Francisco, CA 94080, U.S.A.

Examples of a novel series of phenanthrolinones are shown to be potent competitive inhibitors of avian prolyl 4-hydroxylase, and of collagen hydroxylation, in embryonic chick tendon cells and human foreskin fibroblasts *in vitro* and in the oestradiol-stimulated rat uterus *in vivo*. Two compounds, Compound **1** (1,4-dihydrophenanthrolin-4-one-3-carboxylic acid) and Compound **5** [8-(*N*-butyl-*N*-ethylcarbamoyl)-1,4-dihydrophenathrolin-4-one-3-carboxylic acid], with comparable potencies *in vivo*, were chosen to investigate the effect of the inhibition of the hydroxylation of newly synthesized uterine collagen on the turnover of this protein *in vivo*. Inhibition of hydroxylation by more than 50 % for approx. 8 h following single oral doses of the compounds was associated with significant losses of radiolabelled proline and 4-hydroxyproline from collagen during this period. Progressive hydroxylation of collagen over 48 h, as the inhibitory action of the compounds declined, was accompanied by a decreased loss of radiolabel from the uterine collagen. Earlier reports indicated that underhydroxylated collagen, accumulating within the endoplasmic reticulum in cells where prolyl 4-hydroxylase is inactivated, is slowly degraded, but is then rapidly hydroxylated and secreted when the activity of prolyl 4-hydroxylase is restored. Taken with the present results, this suggests that the potential use of inhibitors of prolyl 4-hydroxylase to control excessive collagen deposition in pathological fibrosis may be limited by the need to maintain continuous inhibition of collagen hydroxylation so as to facilitate intracellular degradation of the accumulated protein.

Key words: collagen accumulation, fibrosis, hydroxylation, turnover.

## INTRODUCTION

There is considerable interest in the potential application of inhibitors of prolyl 4-hydroxylase for the treatment of fibrotic diseases characterized by excessive deposition of collagen, and other connective-tissue elements, leading to the destruction of normal tissue architecture and function [1,2]. The 4-hydroxylation of proline residues in the sequence context -Xaa-Pro-Gly- in collagen chains is essential to the thermal stability of the collagen triple helix. For example, reduction of the 4-hydroxyproline content of Type I collagen by 19 % lowers its thermal-denaturation temperature from the normal body temperature of 37.9 °C to 35.2 °C [3]. Suppression of collagen hydroxylation in cultured cells, either by the depletion of ascorbate [4,5] or by the direct inhibition of prolyl 4-hydroxylase [6,7], results in the visible accumulation of collagen in the endoplasmic reticulum (ER) leading to gross distension of the cisternae of the ER, since incorrectly folded collagen is only slowly degraded or secreted into the external medium. The case for the potential use of inhibitors of prolyl 4-hydroxylase in the treatment in fibrotic diseases rests on the proposition [1] that the resulting underhydroxylated collagen which accumulates in the ER would be degraded *in situ*, within the lysosomal compartment, or by extracellular enzymes in the case of collagen escaping from the cells. However, the marked accumulation of collagen within the ER in cells in which prolyl 4-hydroxylase is inactivated indicates that the procollagen chains fall into the category of incorrectly folded proteins which are only slowly turned over in the ER [8]. Furthermore, the accumulated collagen within the ER is rapidly hydroxylated and secreted from cells when the activity of prolyl 4-hydroxylase is restored [5]. This observation is relevant to the possible use of inhibitors of prolyl 4-hydroxylase to control collagen accumulation *in vivo*, where it may be difficult to maintain continuous inhibition of the enzyme.

We previously reported the ability of novel derivatives of *N*-oxalylglycine to inhibit prolyl 4-hydroxylase *in vivo* [9], although the toxicity of these compounds, which was apparently unrelated to the inhibition of collagen hydroxylation, precluded a more detailed investigation of their activity *in vivo*. We now describe some properties of a novel series of phenanthrolinones, which are potent inhibitors of prolyl 4-hydroxylation both *in vitro* and *in vivo*, and whose safety profile in rats enabled us to examine the effects of underhydroxylation on the turnover of newly synthesized collagen *in vivo* in this species.

## EXPERIMENTAL

### Materials

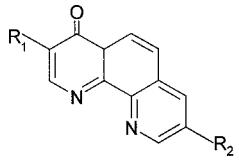
Avian prolyl 4-hydroxylase was purified from the legs of 16-day-old chick embryos [10]. Ascorbic acid (sodium salt), DL-dithiothreitol, catalase, BSA and Tris were all obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Poly(L-Pro-Gly-Pro), average $M_r$ 5200, was purchased from Miles Yeda, Rehovot, Israel, 2-oxo[5-$^{14}$C]glutaric acid (disodium salt, 50 mCi/mmol), L-[U-$^{14}$C]proline (262 mCi/mmol) and L-[4-

Abbreviations used: ER, endoplasmic reticulum; TMB, 3,3′,5,5′-tetramethylbenzidine; HFF, human foreskin fibroblasts; DMEM, Dulbecco's modified Eagle's medium.

[1] To whom correspondence should be sent, at the following address: Silver Trees, Moss Lane, Congleton, Cheshire, CW12 4SX, U.K. (e-mail: trevorfranklin@compuserve.com).

**Table 1 Inhibition of isolated chick prolyl 4-hydroxylase ('Against P4H'), collagen hydroxylation in embryonic chick tendon cells ('Against tendon cells') and procollagen synthesis in HFF cells ('Against HFF cells') by novel phenanthrolinones**

Abbreviations used: Bu, butyl; Et, ethyl; n.d., not determined.



| Compound | $IC_{50}$($\mu$M) | | |
|---|---|---|---|
| | Against P4H | Against tendon cells ($\mu$M) | Against HFF cells |
| **1** ($R_1$=$CO_2H$, $R_2$=H) | 3.6 | 4.7 | 2.4 |
| **2** ($R_1$=$CO_2H$, $R_2$=$CO_2H$) | 0.20 | > 20 | > 20 |
| **3** ($R_1$=H, $R_2$=$CO_2H$) | 1.3 | > 20 | > 20 |
| **4** ($R_1$=$CO_2H$, $R_2$=$CONH_2$) | 0.51 | > 20 | > 20 |
| **5** ($R_1$=$CO_2H$, $R_2$=CONEtBu) | 0.38 | 1.2 | 2.3 |
| **6** ($R_1$=$NO_2$, $R_2$=H) | 0.12 | 0.24 | 1.1 |
| **7** ($R_1$=$NO_2$, $R_2$=$CO_2H$) | 0.038 | > 20 | n.d. |

$^3$H(n)]proline (21 Ci/mmol) were purchased from Amersham International, Amersham, Bucks., U.K. Unlabelled 2-oxoglutaric acid was obtained from Boehringer, Mannheim, Germany, $(NH_4)_2Fe(SO_4)_2$ from BDH Chemicals, Poole, Dorset, U.K., and TMB-ELISA (where TMB refers to 3,3′,5,5′-tetramethylbenzidine) from Gibco Life Technologies.

### Inhibitors of prolyl 4-hydroxylase

Details of the synthesis of compounds of the general formula shown in Table 1 are available from T.J.F. on request.

### Assay of prolyl 4-hydroxylase

The activity of isolated avian prolyl 4-hydroxylase was assayed as previously described [11]. In this method enzyme activity is determined by measuring the conversion of the co-substrate, 2-oxoglutarate, into succinate, a process that stoichiometrically accompanies the hydroxylation reaction. 2-Oxo[5-$^{14}$C]glutarate is separated from the product, [1-$^{14}$C]succinate, on BondElut™ SAX anion-exchange minicolumns (Analytichem International, Harbor City, CA, U.S.A.) after stopping the reaction with phosphate buffer, pH 3.2. The inhibitors, dissolved in DMSO, were added to the enzyme/substrate reaction mixture to give a final DMSO concentration of 0.2%.

### Assay of collagen hydroxylation in cells *in vitro*

#### Embryonic chick tendon cells

Collagen hydroxylation in embryonic-chick tendon cells was determined as previously described [12]. Inhibitors were dissolved in DMSO and the final concentration of this solvent then diluted to 0.5% (v/v) with buffer (enzyme assay) or growth medium (tendon cell assay); this concentration of DMSO had no effect on the activities being measured.

#### Human foreskin fibroblasts (HFF cells)

Collagen hydroxylation in human HFF cells was assayed indirectly by measuring procollagen secretion into the culture medium (which depends on collagen hydroxylation and consequent triple-helix formation) as follows. HFF cells were grown to confluence in 96-well plates at 37 °C in an air/$CO_2$ (9:1) atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal-bovine serum, 100 units/ml of penicillin and 50 $\mu$g/ml of streptomycin. The medium was removed after 24 h and replaced with serum-free DMEM (90 $\mu$l) containing 30 $\mu$g/ml ascorbate and penicillin/streptomycin. The cultures were incubated for a further 24 h, the medium then removed and fresh serum-free medium (90 $\mu$l) containing ascorbate and penicillin/streptomycin was added, followed by the addition of a solution of test compound dissolved in DMEM containing 5% DMSO (10 $\mu$l). After incubation for 48 h, the medium was removed for the assay of procollagen by a standard indirect ELISA assay. The medium was diluted 200-fold into 0.1 M $NaHCO_3$ (pH 10.0) and 100 $\mu$l then used to coat an ELISA plate. The plate was blocked with BSA and then incubated with the primary antibody directed against a peptide sequence from the *N*-propeptide of collagen $\alpha_1$(I). A horseradish-peroxidase-coupled goat anti-rabbit antibody was used as the secondary antibody. The plate was developed for approx. 10 min with TMB-ELISA, the reaction stopped with 1.0 M HCl and the $A_{450}$ measured.

### Assay of collagen hydroxylation *in vivo*

Collagen hydroxylation *in vivo* was determined as previously described [13]. Briefly, 50 g female Sprague–Dawley rats (groups of ten) were dosed subcutaneously on two successive days with oestradiol benzoate (0.5 $\mu$g) to induce rapid collagen synthesis in the uterus. At 24 h after the second dose of oestradiol, the animals were given 5 $\mu$Ci of L-[$^{14}$C]proline dissolved in 0.2 ml of normal saline (0.9% NaCl) by intraperitoneal injection. The animals were humanely killed at the indicated intervals after the administration of [$^{14}$C]proline by exposure to a rising concentration of $CO_2$. The uterus and other tissues were removed into ice-cold 10% (w/v) trichloracetic acid. After successive washes in 10% trichloracetic acid, ethanol and diethyl ether, followed by air drying, the tissues were heated in water at 120 °C for 1 h to extract newly synthesized collagen as gelatin. The gelatin was hydrolysed in 6 M HCl for 16 h and proline and 4-hydroxyproline separated, without prior derivatization, by HPLC on Spherisorb S5ODS1 (Waters Corporation, Milford, MA, U.S.A.) with isocratic elution using a mobile phase consisting of 90% (v/v) water, 9.6% (v/v) propan-2-ol, 0.1% (w/v) trifluoroacetic acid and 0.3% (w/v) SDS. The radioactivity of the proline and 4-hydroxyproline peaks was measured in-line using a Berthold LB506 C-1 counter with an yttrium/glass Y-150 detector. Compounds were given to the rats by oral gavage as ball-milled suspensions in a vehicle containing hydroxypropylmethyl-cellulose (5%) and Polysorbate 80 (1%) at intervals up to 18 h before the administration of radiolabel.

## RESULTS

### Inhibition of prolyl 4-hydroxylase by phenanthrolinones

Approx. 140 phenanthrolinones, of the general formula given in Table 1, were tested for their ability to inhibit purified chick prolyl 4-hydroxylase. The key features of the structure–activity relationships are illustrated by the selected compounds in Table 1. The introduction of a carboxyl group at $R_2$ strongly increased

**Table 2 Effect of the prolyl 4-hydroxylase inhibitor Compound 5 ('5') on the hydroxylation and turnover of newly synthesized uterine collagen *in vivo* over 24 h**

Immature female rats (groups of ten), pretreated with oestradiol (see the Experimental section), were each given a single oral dose of **5** (50 mg/kg) 2 h before an intraperitoneal injection of L-[$^{14}$C]proline (5 $\mu$Ci). The animals were killed at the indicated intervals and the uteri removed for the analysis of the radioactivity associated with the proline and 4-hydroxyproline residues of the newly synthesized collagen. Values are means ± S.E.M. ***$P < 0.001$ versus untreated controls; †††$P < 0.001$ versus **5**-treated animals at 2 h; ‡‡$P < 0.01$ versus **5**-treated animals at 8 h.

| Treatment | Hydroxylation of proline (%) | Total radioactivity (proline + 4-hydroxyproline) (d.p.m.) |
|---|---|---|
| Killed at 2 h after [$^{14}$C]proline | | |
| None | 43.7 ± 0.4 | 3835 ± 356 |
| **5** | 23.3 ± 1.6*** | 3097 ± 170 |
| Killed at 4 h after [$^{14}$C]proline | | |
| None | 42.9 ± 0.6 | 5184 ± 698 |
| **5** | 23.6 ± 1.6*** | 2612 ± 224 |
| Killed at 6 h after [$^{14}$C]proline | | |
| None | 42.4 ± 0.6 | 5604 ± 812 |
| **5** | 23.9 ± 1.6*** | 2567 ± 278 |
| Killed at 8 h after [$^{14}$C]proline | | |
| None | 41.6 ± 0.7 | 5244 ± 507 |
| **5** | 21.2 ± 1.8*** | 1639 ± 196††† |
| Killed at 22 h after [$^{14}$C]proline | | |
| None | 43.3 ± 0.7 | 5196 ± 660 |
| **5** | 28.0 ± 1.4‡‡ | 1701 ± 152††† |

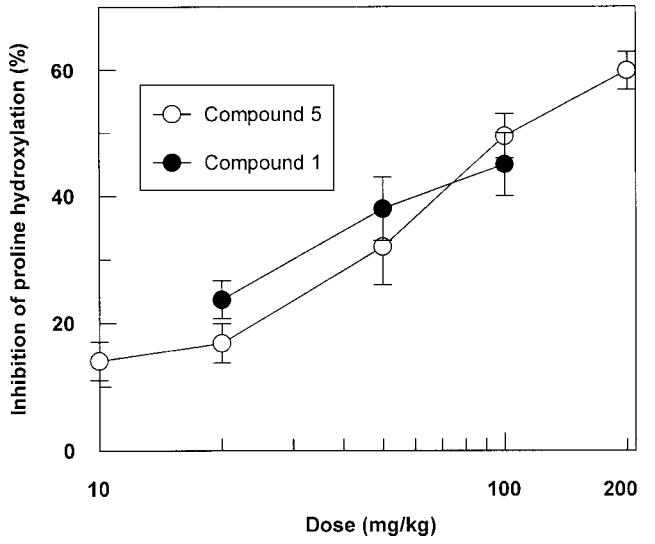



**Figure 1 Dose responses for the inhibition *in vivo* of the hydroxylation of proline residues of newly synthesized uterine collagen in rats 6 h after single oral doses of Compounds 1 and 5**

The compounds were dosed orally and the 4-hydroxylation of proline residues determined as described in the Experimental section. Groups of ten animals were used, and the values are means ± S.E.M.

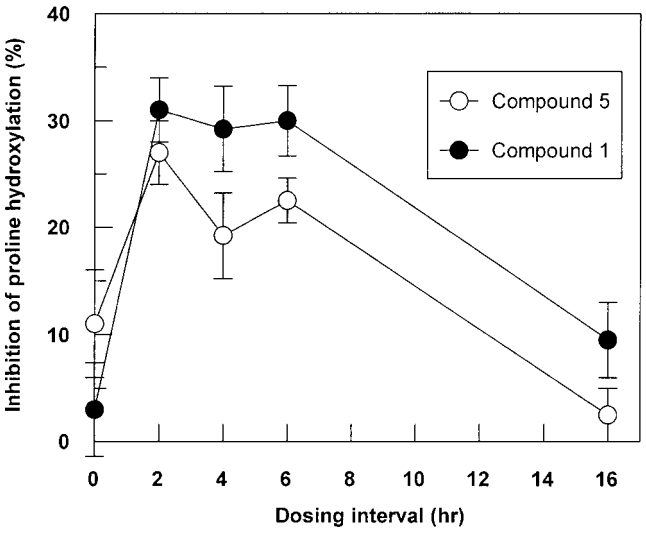



**Figure 2 Time course of the inhibition *in vivo* of the hydroxylation of proline residues of newly synthesized uterine collagen in rats by Compounds 1 and 5**

The compounds were given orally to groups of ten animals at the indicated intervals before the administration of [$^{14}$C]proline ('Dosing interval') and the animals were killed 2 h later. Collagen hydroxylation was determined as described in the Experimental section. Values are means ± S.E.M.

the inhibitory potency, whereas a second carboxy group at $R_1$ also increased potency, although less markedly. The introduction of a nitro function at $R_1$ strongly favoured inhibitory potency, and the combination of a nitro group at $R_1$ and a carboxy group at $R_2$ gave the most potent compound of the series, Compound **7**, against the isolated enzyme. Kinetic analysis of two examples of the series, **1** and **5**, showed that both compounds were competitive inhibitors of 2-oxoglutarate with a $K_i$ values of 0.65 $\mu$M and 16.6 nM respectively. Preliminary studies on the effects of BSA (which is essential for the activity of prolyl 4-hydroxylase *in vitro*) suggest that differential levels of binding of the phenanthrolinones to albumin may contribute to the observed potencies.

### Inhibition by phenanthrolinones of collagen hydroxylation in embryonic chick tendon fibroblasts and of the secretion of procollagen by HFF cells

Although the presence of a carboxy group at position $R_2$ in the phenanthrolinones strongly enhanced inhibition of the isolated enzyme, it is clear from Table 1 that this substitution was markedly unfavourable for inhibition of collagen hydroxylation in both cultured embryonic-chick tendon cells and the secretion of procollagen from HFF cells. The higher activity against intact cells of **1**, which lacks a carboxy group at $R_2$, and of **5**, in which the carboxy group at $R_2$ is replaced by a lipophilic *N*-alkylamide function, suggests that the ionization of the $R_2$ carboxy group at neutral pH may hinder the diffusion of Compound **2**, Compound **3** and Compound **7** to the intracellular location of prolyl 4-hydroxylase within the cisternae of the endoplasmic reticulum. Except in the case of Compound **6**, there were no substantial differences in the inhibitory activity of the compounds against cultured chick tendon cells and HFF cells, despite the much longer period of exposure to compounds of HFF cell assay (48 h compared with 3 h for chick tendon cells).

**1** and its *N*-alkylamide derivative, **5**, were selected for further study *in vivo* because the differencies in their potencies against the isolated enzyme and intact cells were relatively small (Table 1),

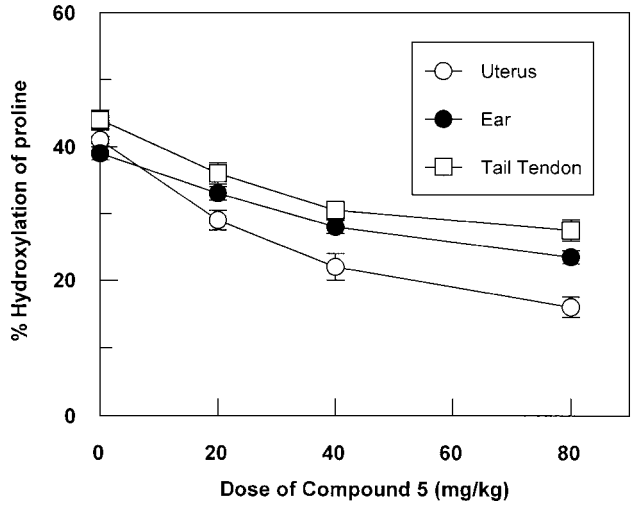



**Figure 3 Inhibition by Compound 5 of the 4-hydroxylation of proline residues of newly synthesized collagen of various tissues *in vivo* in the rat 6 h after the compound was given orally to groups of ten animals**

Collagen hydroxylation was determined as described in the Experimental section. Values are means ± S.E.M.

suggesting facile penetration to the intracellular enzyme. Neither compound affected the level of incorporation of [$^{14}$C]proline into collagen in chick fibroblasts at concentrations of twice their $IC_{50}$ values (results not shown), indicating the absence of non-specific toxicity against these cells. The most potent compound against collagen processing in cultured cells, **6**, was unfortunately too insoluble for *in vivo* studies.

### Inhibition of prolyl 4-hydroxylase *in vivo* by phenanthrolinones

Figure 1 illustrates typical dose responses for the inhibition of collagen hydroxylation in the oestadiol-stimulated rat uterus *in vivo* for **1** and **5** dosed 4 h before giving [$^{14}$C]proline. Despite the greater potency of **5** against intracellular collagen hydroxylation *in vitro*, the inhibitory activities of the two compounds were comparable *in vivo*.

The inhibition of uterine prolyl 4-hydroxylase *in vivo* by both compounds reached a maximum approx. 2 h after oral dosing and remained relatively constant for at least 6 h (Figure 2). These results were consistent with preliminary observations on the plasma concentrations of **1** and **5**, which also reached their maxima 2 h after dosing and remained approximately constant for up to 8 h, falling to unmeasurable levels 16 h after dosing (results not shown).

The ability of **5** to inhibit prolyl hydroxylase *in vivo* was not confined to the uterus. Figure 3 compares the inhibitory effects of the compound on proline hydroxylation in gelatinized, newly synthesized collagen from the uterus and from the external ear and tail tendon. Similar results were obtained with **1** (results not shown).

### Effect of inhibition of prolyl 4-hydroxylase *in vivo* on the turnover of collagen

The inhibition of collagen hydroxylation *in vivo* by **1** and **5** enabled us to assess the effect of incomplete collagen processing on the turnover of newly synthesized uterine collagen. A toxicological study showed that the compounds could be given orally to rats at daily doses up to 80 mg/kg of body weight for 4 days without effects on body-weight gain or general clinical condition and without eliciting histopathological changes in stomach, duodenum, liver, kidneys, lungs, lymph nodes or bone marrow (results not shown).

**Table 3 Effect of the prolyl 4-hydroxylase inhibitor Compound 1 ('1') on the hydroxylation and turnover of newly synthesized uterine collagen *in vivo* over 48 h**

Immature female rats (groups of ten), pretreated with oestradiol (see the Experimental section), were each given an intraperitoneal injection of L-[$^{14}$C]proline (5 $\mu$Ci). As indicated in the Table, some groups received a single oral dose of Compound **1** ('**1**') (5 mg/kg) 2 h before the radiolabel. At 2 h after the radiolabel, some of the animals, as indicated above, were given an intraperitoneal injection of unlabelled L-proline (50 mg/kg). The first set of animals were killed 5 h after [$^{14}$C]proline. Dosing with unlabelled L-proline was repeated for the animals killed at 24 h following [$^{14}$C]proline at 5 and 22.5 h after the radiolabel and at 5, 22.5, 29 and 46.5 h for the animals killed at 48 h after the radiolabel. The uteri were removed for the analysis of radioactivity associated with the proline and 4-hydroxyproline residues of the newly synthesized collagen. Values are means ± S.E.M. Statistical significance: $^{***}P < 0.001$ versus corresponding controls not dosed with **1**; †††$P < 0.001$; ††$P < 0.01$; †$P < 0.05$ versus corresponding treatment groups killed 5 h after [$^{14}$C]proline.

| Treatment | Hydroxylation of proline (%) | Total radioactivity (proline + 4-hydroxyproline) (d.p.m.) |
|---|---|---|
| Killed 5 h after [$^{14}$C]proline | | |
| None | 42.5 ± 0.9 | 9142 ± 699 |
| Unlabelled L-proline (one dose) | 41.7 ± 0.6 | 7508 ± 629 |
| **1** | 12.1 ± 1.4*** | 2979 ± 549 |
| **1** + unlabelled L-proline (one dose) | 13.9 ± 1.2*** | 3836 ± 525 |
| Killed 24 h after [$^{14}$C]proline | | |
| None | 46.8 ± 0.8 | 7829 ± 662 |
| Unlabelled L-proline (three doses) | 45.0 ± 1.1 | 7402 ± 1086 |
| **1** | 25.9 ± 2.2*** | 1621 ± 166† |
| **1** + unlabelled L-proline (three doses) | 29.4 ± 1.1*** | 1857 ± 516††† |
| Killed 48 h after [$^{14}$C]proline | | |
| None | 47.2 ± 0.5 | 6920 ± 730 |
| Unlabelled L-proline (five doses) | 46.9 ± 0.4 | 7843 ± 1275 |
| **1** | 36.3 ± 1.7*** | 1901 ± 198† |
| **1** + unlabelled L-proline (five doses) | 35.6 ± 0.9*** | 2022 ± 270†† |

In the first experiment, **5** (50 mg/kg) was given orally to oestradiol-treated immature females rats 2 h before the administration of [$^{14}$C]proline. The animals were killed at 2, 4, 6, 8 or 22 h after the radiolabel and the hydroxylation of proline, together with the total incorporation of radiolabel into proline and 4-hydroxyproline of the newly synthesized uterine collagen determined (Table 2). The compound inhibited hydroxylation by 46 % at 2 h after the radiolabel and this level of inhibition was sustained over 8 h. Hydroxylation then increased significantly by 22 h ($P < 0.01$ versus the 8 h value). The combined incorporation of radiolabel into proline and 4-hydroxyproline was maximal in undosed animals 6 h after giving the radiolabel and remained relatively constant until the end of the experiment. In animals dosed with **5** the total radioactivity incorporated was maximal by 2 h following the radiolabel, declined until 8 h and then remained constant until at least 22 h

A further experiment was performed to investigate the effect of the inhibition of prolyl 4-hydroxylase on the fate of radiolabelled proline incorporated into newly synthesized uterine collagen over 48 h. Because the pharmacodynamic activity of **1** over 16 h was apparently somewhat greater than that of **5** (Figure 2), it was decided to dose the animals with **1** in the 48 h experiment in the expectation of a more definitive outcome than with **5**. A single

dose of **1** (50 mg/kg) was given to rats 2 h before [$^{14}$C]proline and the uteri removed from groups of animals killed 5, 24 and 48 h after the radiolabel. Because reincorporation into collagen of [$^{14}$C]proline released by protein turnover may lead to an overestimate of the stability of uterine collagen, unlabelled L-proline (50 mg/kg) was given as a solution in normal saline by intraperitoneal injection to groups of rats 2, 5, 22.5, 29 and 46.5 h after [$^{14}$C]proline. Table 3 indicates that, 5 h after dosing, **1** inhibited the hydroxylation of newly synthesized collagen by approx. 70 %. The level of inhibition then declined, falling to approx. 15 % inhibition 48 h after giving the radiolabel (i.e. 50 h after giving **1**), indicating that the underhydroxylated collagen was progressively hydroxylated as the tissue level of **1** fell. The total radioactivity of the newly synthesized collagen of the control group fell by 24.3 % over 48 h. The decline in radioactivity was not increased by the repeated administration of unlabelled L-proline, indicating that there was minimal re-incorporation of [$^{14}$C]proline released by protein turnover. In animals dosed with **1** there was a highly significant loss (46 %) of radioactivity from uterine collagen in the period 5–24 h following the administration of radiolabel. This decline was also unaffected by giving animals unlabelled proline. No further decline in total radioactivity was observed in animals dosed with **1** in the period 24–48 h following the radiolabel.

## DISCUSSION

We previously described the ability of a novel series of heterocyclic carbonylglycines to inhibit prolyl 4-hydroxylase *in vivo* in the rat [9]. Unfortunately, the toxicity of these compounds, which was unrelated to their inhibition of prolyl 4-hydroxylase, precluded investigation of their effects on the stability of collagen *in vivo*. We have now described the inhibitory activity of members of a structurally different series of novel compounds, the phenanthrolinones, against purified chick prolyl 4-hydroxylase and also against collagen hydroxylation in cultured embryonic-chick tendon cells and against collagen secretion from human foreskin fibroblasts *in vitro*. Recombinant human prolyl 4-hydroxylase is also inhibited by selected phenanthrolinones (M. Brenner, personal communication). Two phenanthrolinones, which are competitive antagonists of the co-substrate, 2-oxoglutarate, were well tolerated in the rat at doses producing sustained inhibition of collagen hydroxylation in several tissues *in vivo*.

On the basis of data indicating that a modest reduction in the 4-hydroxyproline content of collagen results in a decrease in the thermal-denaturation temperature of the triple helix below the normal body temperature [3], we anticipated that inhibition of prolyl hydroxylase *in vivo* would result in an accelerated collagen turnover due to intracellular degradation of the underprocessed collagen. There is evidence that misfolded or incorrectly assembled oligomeric complexes are degraded, either within the ER itself [8] or after transport into the lysosomal compartment [14]. However, there is also evidence from *in vitro* systems that underhydroxylated collagen, accumulates in the ER: (1) procollagens Types I and III (detected by specific antibodies) accumulated in the ER of palatal mesenchymal cells from embryonic mice when the cells were cultured for up to 7 days in ascorbate-depleted medium [4]; (2) a marked dilatation of the ER was observed in the cells of embryonic-chicken calvaria cultured in the presence of an inhibitor of prolyl 4-hydroxylase [7]; the dilatation of the ER in this case was also ascribed to an accumulation of underhydroxylated collagen; (3) large amounts of underhydroxylated Type II collagen accumulated within the ER of embryonic-chick chondrocytes maintained for up to 9 days in ascorbate-depleted culture [5]. Addition of ascorbate to the culture medium resulted in hydroxylation of the accumulated collagen within 1 h accompanied by a marked increase in the rate of secretion of fully processed collagen from the chondrocytes within 2–3 h. These reports suggest that the rate of degradation of underhydroxylated collagen chains within the ER is less than the rate of collagen synthesis. Significantly, however, the collagen accumulated within the ER is rapidly hydroxylated and secreted once the activity of prolyl 4-hydroxylase is restored [4,5].

We found that newly synthesized uterine collagen, which was substantially underhydroxylated during the first few hours following dosing with inhibitors of prolyl 4-hydroxylase, was turned over more rapidly than normal collagen, as indicated by a marked loss of the radioactivity associated with proline and 4-hydroxyproline residues. The hydroxylation of proline residues was then gradually restored over the next 40 h, presumably as the uterine concentration of inhibitor declined. The gradual increase in the 4-hydroxyproline content of the uterine collagen coincided with a reduced turnover of the newly synthesized collagen, although whether these two events are causally linked remains uncertain at present.

The *in vivo* data presented here, together with those from earlier reports of *in vitro* systems, may have implications for the potential use of inhibitors of prolyl 4-hydroxylase in the treatment of life-threatening progressive fibrotic disorders of the liver, lungs, kidneys and other tissues. Because underhydroxylated collagen only accumulates in the ER when prolyl 4-hydroxylase is continuously inactivated, the prospect of achieving a worthwhile reduction in the pathological deposition of collagen, resulting from the intracellular degradation of the accumulated protein, may depend on maintaining continuous inhibition of the prolyl hydroxylase with long-acting agents. **1** inhibits collagen accumulation in a model of dermal scarring in the rat (V. Guenzler, personal communication) and currently other phenanthrolinones are being evaluated as potential agents for the prevention of human dermal scarring.

## REFERENCES


1 Hanauske-Abel, H. M. (1991) Prolyl 4-hydroxylase, a target for drug development. J. Hepatol. **13** (Suppl. 3), S8–S16

2 Franklin, T. J. (1997) Therapeutic approaches to organ fibrosis. Int. J. Biochem. Cell Biol. **79**, 79–89

3 Rosenbloom, J. and Harsch, M. (1973) Hydroxyproline content determines denaturation temperature of chick tendon collagen. Arch. Biochem. Biophys. **158**, 478–484

4 Kurisu, K., Ohsaki, Y., Nagata, K., Inai, T. and Kukita, Y. (1992) Heterogeneous distribution of the precursor of type I and type III collagen and fibronectin in the rough endoplasmic reticulum of palatal mesenchymal cells of the mouse embryo cultured in ascorbate-depleted medium. Cell Tissue Res. **267**, 429–435

5 Pacifici, M. and Iozzo, R. V. (1988) Remodelling of the rough endoplasmic reticulum during stimulation of procollagen secretion by ascorbic acid in cultured chondrocytes. J. Biol. Chem. **263**, 2483–2492

6 Jimenez, S. A. and Yankowski, R. (1978) Role of molecular conformation on secretion of chick tendon collagen. J. Biol. Chem. **253**, 1420–1426

7 Tschank, G., Brocks, D. G., Engelbart, K., Mohr, J., Baader, E., Guenzler, V. and Hanauske-Abel, H. M. (1991) Inhibition of prolyl hydroxylation and procollagen processing in chick embryo calvaria by a derivative of pyridine-2, 4-dicarboxylate. Biochem. J. **275**, 469–476

8 Klausner, R. D. and Sitia, R. (1990) Protein degradation in the endoplasmic reticulum. Cell **62**, 611–614

9 Franklin, T. J., Hales, N. J., Johnstone, D., Morris, W. P., Cunliffe, C. J., Millest, A. J. and Hill, G. B. (1991) Approaches to the design of antifibrotic drugs. Biochem. Soc. Trans. **19**, 812–815

10 Kedersha, N. L. and Berg, R. A. (1981) An improved method for the purification of vertebrate prolyl hydroxylase by affinity chomatography. Collagen Relat. Res. **1**, 345–353

11 Cunliffe, C. J., Franklin, T. J. and Gaskell, R. M. (1986) Assay of prolyl 4-hydroxylase by the chomatographic determination of [$^{14}$C]succinic acid on ion-exchange minicolumns. Biochem. J. **240**, 617–619
12 Franklin, T. J. and Hitchen, M. (1989) Inhibition of collagen hydroxylation by 2,7,8-trihydroxyanthraquinone in embryonic-chick tendon cells. Biochem. J. **261**, 127–130
13 Franklin, T. J., Morris, W. P., Gaskell, R. M. and Millest, A. J. (1990) Rapid method for the determination of the 4-hydroxylation of proline *in vivo*. Matrix **10**, 239
14 Berg, R. A., Schwartz, M. L. and Crystal, R. G. (1980) Regulation of the production of secretory proteins: intracellular degradation of newly synthesised "defective" collagen. Proc. Natl. Acad. Sci. U.S.A. **77**, 4746–4750

Received 11 September 2000/16 October 2000; accepted 7 November 2000