

Approaches to the design of anti-fibrotic drugs

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Introduction

Progressive fibro-proliferative diseases (FPDs), such as liver cirrhosis, pulmonary fibrosis, scleroderma, ankylosing spondylitis and rheumatoid arthritis, exhibit excessive production of connective tissue and the resulting destruction of normal tissue architecture and function. It is generally recognized that most treatments for these diseases have little effect upon the inexorable pathological progression. Elimination of the initiating cause, where it is known, can be helpful. For example, abstinence from alcohol improves survival in liver cirrhosis [1] and removal of patients with early silicosis of the lungs from the silica-dust-laden environment has long been known to be of major benefit. In cases of liver fibrosis resulting from chronic infection with hepatitis C, suppression of viral activity by interferon- α appears to slow the fibrogenic process in a proportion of patients [2]. In most FPDs a chronic inflammatory process may provide a continuing stimulus to fibrogenesis. Treatment of alcoholic liver disease with anti-inflammatory steroids benefits some patients [1] and this form of treatment is occasionally dramatically successful for scleroderma and pulmonary fibrosis [3]. Anti-inflammatory therapy of joint disease generally provides only symptomatic relief.

The possibility of a direct attack on the growth and proliferation of the connective tissue has been raised many times. Cytotoxic drugs have been used in pulmonary fibrosis in the hope of slowing the proliferation of fibroblasts [4]. These drugs are also used in the treatment of rheumatoid arthritis, and methotrexate, for example, shows particular promise although its mode of action is uncertain and may involve suppression of some aspects of immune function [5]. There is increasing interest in the possibility of modulating the functions of cytokines, believed to regulate the proliferative and biosynthetic activities of connective tissue [6]. These include transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and interleukin-1. However, there are formidable technical obstacles to be overcome in the design and development of

therapeutically useful antagonists of cytokine function.

Attempts have been made at the direct control of collagen synthesis and secretion since excessive accumulation of collagen types I and III is prominent in all FPDs. Colchicine, which slows the secretion of collagen into the extracellular matrix (ECM), has been used with some apparent success in extended clinical trials to limit the fibrotic process in liver cirrhosis [7]. In experimental models of fibrosis, compounds such as β -aminopropionitrile, an inhibitor of the collagen cross-linking enzyme, lysyl oxidase EC 1.4.3.14, and various analogues of proline, including 3,4-dehydropyrrolidine and *cis*-hydroxyproline, all depress the accumulation of collagen [8]. Unfortunately, these anti-collagen compounds are insufficiently specific to permit sustained clinical use in human fibrotic disease. A more specific agent is therefore required.

Inhibition of prolyl 4-hydroxylase

We have concentrated on the design of inhibitors of prolyl 4-hydroxylase EC 1.14.11.2. The activity of this enzyme is essential for the formation of the triple helices of collagen and its subsequent secretion into the ECM [9]. Prolyl 4-hydroxylase belongs to a family of Fe^{2+} -dependent di-oxygenases which couple the oxidative decarboxylation of 2-oxoglutarate to the hydroxylation of the primary substrate. In the case of prolyl 4-hydroxylase the targets of hydroxylation are prolyl residues in the sequence context -X-Pro-Gly- in procollagen chains. The di-oxygenases use molecular oxygen as the third substrate and require ascorbate as a co-factor.

Consideration of the likely catalytic mechanism of prolyl 4-hydroxylase [10] suggests several possible approaches to the design of inhibitors: (1) Competition with procollagen. The low K_m for procollagen ($0.2 \mu\text{M}$) indicated that this would be a difficult undertaking, probably requiring a modified oligopeptide to achieve a useful level of competition. (2) Competition with ascorbate. The possibility of non-specific interference with the diverse functions of ascorbate made this an unattractive option. (3) Removal of the essential Fe^{2+} by chelation. The well-known iron-chelator 2,2'-dipyridyl is known to inhibit prolyl hydroxylase

Abbreviations used: FPD, fibro-proliferative disease; ECM, extracellular matrix.

in this way but the likelihood of undesirable side-effects owing to iron depletion rule out this approach. (4) Competition with 2-oxoglutarate. Pyridyl 2,4- and 2,5-dicarboxylic acids inhibit the isolated enzyme by competition with 2-oxoglutarate [11]. Unfortunately the ionization of the C-groups at physiological pH prevents these compounds from gaining access to the intracellular location of the enzyme in the endoplasmic reticulum [12]. This problem has been addressed by preparing ester and amide derivatives, designed to diffuse more freely across the cytoplasmic membrane and then to be degraded by intracellular esterases or amidases, to release the active pyridyl dicarboxylates within the cell [13]. So far, however, there is no direct evidence that such 'prodrugs' provide effective inhibition of prolyl 4-hydroxylase *in vivo*.

We have pursued several approaches to the antagonism of 2-oxoglutarate in the search for effective inhibitors of prolyl 4-hydroxylase *in vivo*,

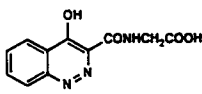
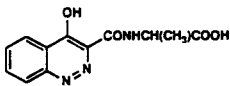
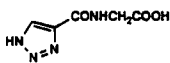
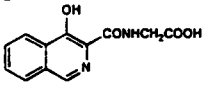
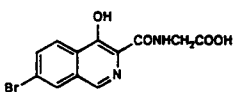
including the exploration of hydroxyanthraquinones [14], substituted pyridyl 2- β -diketones (C. J. Cunliffe, R. I. Dowell, T. J. Franklin, E. M. Hadley & M. D. Hitchen, unpublished work) and pyridyl 2-carboxyl, 5-acylsulphonamide derivatives (R. I. Dowell & E. M. Hadley, unpublished work). However, although interesting activity was found against both the isolated enzyme and against collagen hydroxylation in suspensions of intact tendon cells, activity *in vivo* among these series continued to elude us.

Derivatives of N-oxalylglycine as inhibitors of prolyl 4-hydroxylase

Inspection of the structure of 2-oxoglutarate suggested the analogous compound, N-oxalylglycine, as another possible antagonist of the enzyme. Kinetic analysis showed that oxalylglycine is indeed a competitive inhibitor of prolyl hydroxylase with a K_i of 0.54 μM . Substitutions at the α -carbon atom all

Table I
Inhibition of prolyl 4-hydroxylase *in vitro* by N-oxalylglycine and its derivatives

IC₅₀ refers to the concentration required to inhibit the indicated activity by 50%.

Compound	IC ₅₀ (μM)	
	Prolyl 4-hydroxylase	Prolyl 4-hydroxylation in isolated chick-tendon cells
N-Oxalylglycine	3	> 150
N-Oxalylalanine (S)	48	
N-Oxalylalanine (R)	621	
N-Oxalylsarcosine	310	
N-Oxalylphenylalanine	211	
A	0.02	> 81
		
A1: S-form A2: R-form	0.16(S), 3(R)	
		
B	22	> 130
		
C	0.19	20
		
D	0.98	15
		

We think it possible that the heterocyclic derivatives of *N*-oxalylglycine (heterocyclic carbonylglycines) may co-ordinate the postulated Fe^{2+} at the active centre of the enzyme through the nitrogen atom of the heterocyclic ring *ortho* to the side-chain and through the amide carbonyl group (Fig. 1). A free C-group is also necessary for inhibition (data not shown) and this may interact with a complementary positively charged amino-acid side-chain in the enzyme.

The ability of oxalylglycine and its derivatives to inhibit the 4-hydroxylation of collagenous prolyl residues in isolated embryonic-chick-tendon cells was assessed as previously described [15]. Oxalyl-

Suggested mode of interaction of heterocyclic carbonylglycines with the active centre of prolyl 4-hydroxylase

The ability of compounds to inhibit the enzyme *in vivo* in the rat was assessed as previously described [16]. Compounds were given orally 2 h before the administration of [^{14}C]proline and the animals were killed 2 h after the radiolabel. Table 2 shows that oxyalylglycine (200 mg/kg of body weight) had no effect on the 4-hydroxylation of prolyl residues in the uterine gelatin fraction. Of the heterocyclic carbonylglycines tested compound A was inactive, while both C and D gave significant inhibition. Compound D was by far the more active and significant inhibition was observed after a dose of 25 mg/kg. Furthermore, inhibition was evident for at least 24 h after a single oral dose of 100 mg/kg. We believe that the marked difference in the activities *in vivo* of compounds C and D may be due to the protection against possible metabolic inactivation afforded by the 7-bromo function although this remains to be established directly.

Table 2 shows the ability of compounds C and D to inhibit the accumulation of uterine collagen, induced by the administration of oestradiol to immature female rats [17]. Compound C produced only minor inhibition, consistent with its limited inhibition of uterine prolyl 4-hydroxylase. In contrast, compound D caused marked suppression of collagen accumulation and we suggest that this is associated with the ability of this compound to give sustained inhibition of prolyl 4-hydroxylation *in vivo*. Presumably the underhydroxylated collagen

Table 2**Inhibition of prolyl 4-hydroxylase and collagen accumulation *in vivo* by derivatives of *N*-oxalylglycine**

Enzyme activity and collagen accumulation were measured in the uteri of oestradiol-treated, immature female rats (50–70 g). Compounds were dosed orally, singly for the measurement of inhibition of enzyme activity and twice daily for 5 days for the measurement of inhibition of collagen accumulation. L-[U-¹⁴C]Proline (262 mCi/mmol) was given by intraperitoneal injection, 5 μ Ci/rat. Statistical significance: $n = 10$, *** $P > 0.001$; ** $P > 0.01$; * $P > 0.05$.

Compound	Dose (mg/kg of body weight)	Inhibition of prolyl hydroxylase (%)	Inhibition of collagen accumulation (%)
<i>N</i> -Oxalylglycine	200	0	—
Compound A	200	0	23.1
Compound C	200	27.6*	23.1
Compound D	200	83.4***	—
Compound D	100	71.5***	—
Compound D	50	48.0***	100.0***
Compound D	25	20.2***	75.0***
Compound D: 2 h before proline	100	83.0***	—
Compound D: 6 h before proline	100	67.9***	—
Compound D: 24 h before proline	100	11.2**	—

formed in the presence of the inhibitor is eventually degraded by intracellular protease activity resulting in a deficit of mature hydroxylated collagen.

Although the further development of compound E was precluded by toxicity, apparently unrelated to the inhibition of prolyl 4-hydroxylase, we believe that inhibitors of this enzyme which are free of non-specific toxicity may have potential for the treatment of life-threatening and disabling diseases characterized by the pathological deposition of collagenous ECM.

We are grateful to Ian Fielding, Adam Houghton and Caroline Bailey for assistance with parts of this work.

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Received 25 July 1991