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Hypothesis

Pyrroloquinoline quinone and molecules mimicking its functional domains

Modulators of connective tissue formation?

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1. INTRODUCTION

The formation of the connective tissue matrix requires the ordered interaction of multiple macromolecular components, most notably the fibrillar and non-fibrillar collagens, elastin, proteoglycans, glycosaminoglycans, and glycoproteins like fibronectin or laminin [1]. As each of these cellular products displays specific binding sites for association with identical or non-identical matrix molecules, or with an arrangement already formed by them, the interaction of these components occurs as a self-organizing process, as exemplified by the auto-generation of highly ordered collagen fibrils from type I collagen molecules [2].

This self-organizing process can be controlled most efficiently by regulating the availability of the individual components. Procollagen peptides, for example, have been reported to suppress collagen biosynthesis at both the pre-translational and translational levels [3] and to inhibit fibronectin biosynthesis post-transcriptionally [4]. In addition to such feed-back mechanisms that govern matrix formation via the *production* of individual components, experimental evidence also suggests that the *processing* of the individual components' precursors may be modulated to alter the structure and function of connective tissue: while matrix accumulation of elastin is strikingly reduced by *increasing* its hydroxyproline content [5–7], that of fibrillar collagen is strikingly reduced by *decreasing* it [8]. Obviously, hydroxyproline has antagonistic functions in these major matrix components. In elastin, it interferes with fiber formation, i.e. coacervation, at 37°C [9] and subsequent cross-link synthesis [5]; in collagen, it is indispensible for fiber formation – the stability of the individual molecule's triple helix as well as the secretion of collagen at 37°C [8] depend on prolyl hydroxylation.

In these two classes of structural proteins which simultaneously can be synthesized by the same cell population [10,11], each processing event – peptidyl hydroxyproline formation and cross-link-stabilized matrix accumulation – is catalyzed by a specific enzyme, the intracellularly active prolyl 4-hydroxylase [12] and the extracellularly active lysyl oxidase [13], respectively. Both enzymes utilize unmodified elastin and unmodified collagen [12–15]; experimental evidence for peptide

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substrate-specific isoenzymes is not available. Thus, the opposing effect of hydroxyprolyl in elastin and in collagen on their matrix accumulation necessitates a modulating mechanism that directs the two enzymes' activity towards a differential processing of each of these essential connective tissue components, a processing that is prerequisite to their physiologically useful deposition. Any modulation of their hydroxyproline content must result in the formation of a highly inhomogeneous, yet ordered pericellular matrix; vice versa, the morphologically complex connective tissue synthesized by certain cells, e.g. smooth muscle cells [1], could in part reflect the time course of the differential activity of prolyl 4-hydroxylase and lysyl oxidase. Molecules able to modulate both enzymes may be indispensible to generate, at a given rate of protein biosynthesis, such a structurally and functionally differentiated pericellular connective tissue. Their identification would allow additional insight into the physiology and pathophysiology of collagen and elastin biosynthesis and contribute to present efforts towards a molecular pharmacology of the extracellular matrix.

Recently, substances were described that affect the activity either of prolyl 4-hydroxylase or of lysyl oxidase. The structural homology displayed by these compounds deserves attention.

Designed and *predicted* to interfere with the decarboxylation phase of the catalytic cycle of prolyl 4-hydroxylase as formulated by Hanauske-Abel and Günzler, HAG [16,32], compounds like 3,4-dihydroxyphenylacetate (3,4-DHPA) and pyridine-2,4-dicarboxylate (2,4-PDCA), representative structures for class IV and class V inhibitors of the HAG mechanism [16], were immediately found to be the enzyme's most potent reversible inhibitors presently available [17–19]. 2,4-PDCA is competitive with respect to 2-oxoglutarate [17] and 3,4-DHPA competitive with respect to 2-oxoglutarate and ascorbate [19], the two co-substrates of prolyl 4-hydroxylase [12,20]. 2,4-PDCA was demonstrated to select for the 2-oxoglutarate-binding site of only the collagen hydroxylases [18] and to inhibit specifically the biosynthesis of hydroxyproline-dependent proteins, i.e. of fibrillar collagens and of C1q [21,32]. According to the pharmacokinetic model of procaine/procainamide, Hanauske-Abel designed organ-targeted lipophilic bioactivatable pro-inhibitors of 2,4-PDCA that, in carbon tetrachloride-induced liver fibrosis, were found to preserve strikingly organ function by virtue of the specific action [46].

Identified as a *natural* compound which is the cofactor of oxidoreductases in methylotropic bacteria [22,23], pyrroloquinoline quinone (PQQ) was suggested [13,24] and subsequently found [25,26] to be the covalently bound prosthetic group of lysyl oxidase. The *ortho* quinone arrangement at PQQ's C4-C5 was demonstrated to participate directly in the function of serum amine oxidase, the first quinoprotein identified in mammals [27,28,44,46].

We argue that the structural homology between the experimental inhibitors of prolyl 4-hydroxylase and the natural cofactor of lysyl oxidase (figs 1,2) should not be dismissed as sheer coincidence, but should encourage attempts to investigate their potential role as physiological or pharmacological modulators of matrix formation.

2. HYPOTHESIS

Formation of hydroxyprolyl, i.e. conversion of primary structure defined proline to *trans* 4-hydroxyproline residues, and formation of functional lysyl oxidase, i.e. covalent attachment of PQQ to lysyl oxidase apoenzyme, occur within the subcellular compartments that constitute the unidirectional route for secretory traffic of de novo synthesized macromolecules, i.e. the cisternae of the rough endoplasmic reticulum and the Golgi complex. Prolyl 4-hydroxylase and the lysyl oxidase-specific PQQ ligase are not differentially accessible for the low-M_r compounds subsequently specified.

PQQ reversibly inhibits prolyl 4-hydroxylase. The mode of inhibition is competitive with respect to at least one of its co-substrates. The C9 carboxyl function and the C4-C5 *ortho* quinone moiety are the structural prerequisites for attachment at the co-substrate-binding site of the enzyme. The compound interferes with cellular peptidyl hydroxyproline formation as an ascorbate antagonist and favors cross-link-stabilized matrix accumulation.

Molecules that mimick PQQ's planar structure and the arrangement of its carboxyl groups reversibly inhibit the lysyl oxidase-specific PQQ ligase,

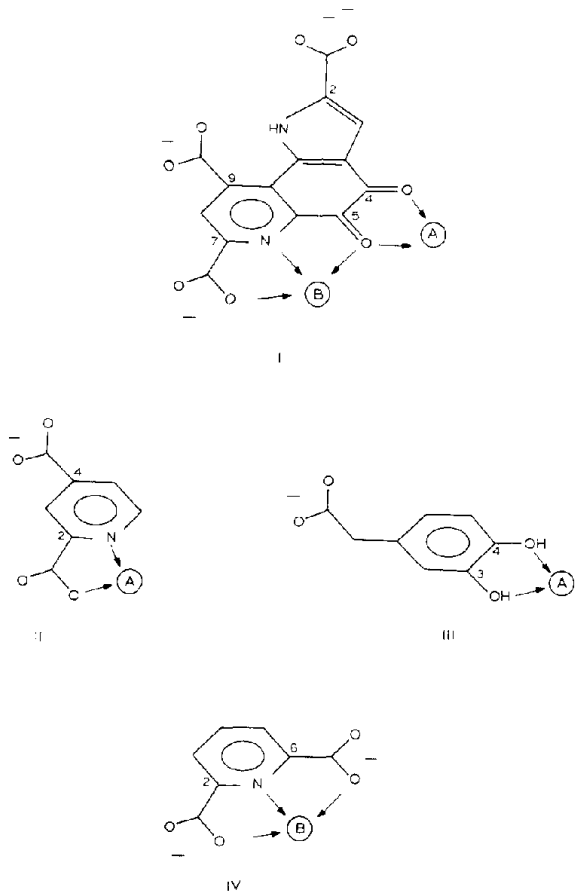

Fig.1. Structural homology between the covalently bound prosthetic group of lysyl oxidase, PQQ (I), and the prolyl 4-hydroxylase inhibitors that act either as specific co-substrate antagonists, i.e. pyridine-2,4-dicarboxylate (II) and 3,4-dihydroxyphenylacetate (III), or as non-specific iron chelators, like pyridine-2,6-dicarboxylate (IV). As probes of the enzyme's mechanism, II and III were designed to interfere with the catalytic cycle of prolyl 4-hydroxylase [16,32] and subsequently demonstrated to be the most efficient inhibitors available [17–19]. The two potential interactions of PQQ with iron atoms are indicated by the encircled 'A' and 'B'. 'A' designates the bidentate mode of coordination to the catalytic site Fe^{2+} of prolyl 4-hydroxylase that is indispensible for effective and specific inhibition; 'B' designates the terdentate mode of coordination that is not possible at this enzyme's co-substrate-binding site and that can occur only in solution, resulting in non-specific inhibition [17–19,43]. PQQ displays II, III and IV as substructures.

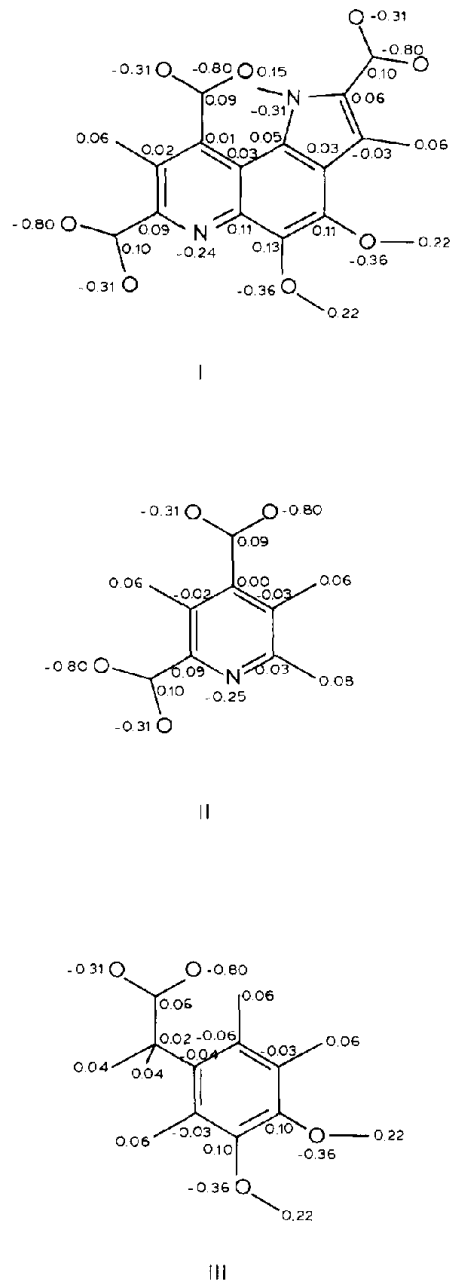

Fig.2. Homology of charge distribution between the reduced form of PQQ, $PQQH_2$ (I) on the one hand and pyridine-2,4-dicarboxylate (II) and 3,4-dihydroxyphenylacetate (III) on the other. The II-like and III-like substructures of I are electronically identical with II and III, respectively. Atomic charges were calculated by the method of Gasteiger and Marsili [42].

in this way interfering with the de novo biosynthesis of functional lysyl oxidase. 2,4-PDCA is the pilot structure for such PQQ ligase inhibitors. This compound suppresses peptidyl hydroxyproline formation as a 2-oxoglutarate antagonist and, by interfering with lysyl oxidase biosynthesis as a PQQ antagonist, reduces cross-link-stabilized matrix accumulation. In this way, two crucial events in the matrix, deposition of both elastin and collagen, are rendered inefficient (elastofibrosuppressive effect).

Molecules that mimick PQQ's C9 carboxyl/C4-C5 *ortho* quinone substructure reversibly inhibit prolyl 4-hydroxylase, competitive to at least one of its co-substrates. The catecholamine-derived compounds 3,4-dihydroxymandelate and 3,4-dihydroxyphenylacetate are the pilot structures for such prolyl 4-hydroxylase inhibitors. These compounds inhibit peptidyl hydroxyproline formation as ascorbate antagonists (fibrosuppressive effect), but do not affect the lysyl oxidase-specific PQQ ligase and consequently cross-link stabilized matrix accumulation. In vivo, both substances can function as physiological modulators of matrix formation, favoring the cross-link-stabilized matrix accumulation of elastin and retarding that of collagen.

3. DISCUSSION

The assumption that the formation of hydroxyprolyl and functional lysyl oxidase is located along the secretory pathway of macromolecule biosynthesis does not contradict any available experimental evidence. Prolyl 4-hydroxylase occurs within the cisternae of the rough-surfaced endoplasmic reticulum (RER) and thus is co-distributed with its collagen and elastin substrates, proteins synthesized for export [8]. Lysyl oxidase, active only in the extracellular space, is a secretory enzyme and as such will also be subject to vectorial transport [29], obviously as a high-M_r precursor [13]. There is, however, a lack of detailed information on the biosynthetic route of lysyl oxidase [13]. The finding that covalently bound PQQ constitutes its prosthetic group [25,26] implies the existence of a specific transporter that serves to import the trianionic PQQ into the cisternae of the RER/Golgi system and of an intracisternal transferase that catalyses PQQ attachment to lysyl oxidase apoenzyme. The absence of a spontaneously reactive moiety in PQQ and its occurrence in just a few proteins [25,26,30,31,46] argue against a non-discriminating non-enzymatic attachment and justify the assumption of a PQQ ligase.

PQQ, in its oxidized (fig.1) or reduced (fig.2) form, displays an essential structural and electronic homology with the representative compounds for the two classes of competitive collagen hydroxylase inhibitors, 3,4-DHPA and 2,4-PDCA. This homology identifies the atom arrangement anticipated to mediate attachment to the co-substrate-binding site of prolyl 4-hydroxylase. According to the HAG mechanism, 2-oxoglutarate antagonists display two functional domains [16,32]: subsite I for electrostatic interaction with a positively charged side chain at the active site; and subsite II for bidentate ligation to the catalytic center's d^2sp^3 hybridized Fe^{2+} that is coordinated, in *cis* arrangement, via three apoenzyme residues. The specific co-substrate antagonists 3,4-DHPA and 2,4-PDCA show these functional domains, while pyridine-2,6-dicarboxylate (2,6-PDCA), which only acts as a terdentate chelator [17,43], does not (fig.1). That is why the 2,6-PDCA-like substructure of PQQ cannot contribute to specific prolyl 4-hydroxylase inhibition; this effect is predicted to result from PQQ's 3,4-DHPA-like substructure (cf. fig.1, I and III). As the co-substrate-binding pocket is able to accommodate inhibitors as large as doxorubicin [33], the overall size of PQQ should not hamper its attachment. Previously, 3,4-DHPA and its analogue 3,4-dihydroxymandelate (3,4-DHMA) were found to inhibit competitively prolyl 4-hydroxylase with respect to its two co-substrates. While their K_i values and the K_m for 2-oxoglutarate are in the same range – about 10 μM – these values differ significantly with respect to ascorbate: the K_i values are at 10 μM, while the K_m for ascorbate is 360 μM [19]. Obviously, 3,4-DHPA and 3,4-DHMA are potent ascorbate antagonists in the prolyl 4-hydroxylase assay. In the same experimental setting, it is reasonable to expect that, due to its 3,4-DHPA-like substructure, PQQ preferentially will act as an ascorbate antagonist. An easily detectable ascorbate agonist function of reduced PQQ is unlikely, due to at least the instability of $PQQH_2$ in aqueous oxygenated media above pH 4

[34]; in addition, none of the *ortho*-dihydroxyphenyl derivatives competitive with respect to ascorbate was able to function as a reductant for the purified enzyme in vitro [19].

It has been suggested that PQQ is the organic cofactor of all Cu-dependent O_2-linked amine oxidases [46]. In these enzymes, its 2,6-PDCA-like substructure is anticipated to contribute to the active-site chelation of the transition metal [47] that is indispensible for any catalytic utilization of molecular oxygen [48].

Release of PQQ has been demonstrated after extensive proteolytic treatment of lysyl oxidase [25], indicating that at least one of its three carboxyl moieties is bound via an enzymatically hydrolysable bond to an appropriate side chain of lysyl oxidase. This finding supports the prediction that the specific ligase requires a distinct arrangement of carboxyl groups around a planar molecule for attachment at its PQQ binding site. 2,4-PDCA fulfills this deduced structural prerequisite but 3,4-DHPA does not: it lacks planarity and distinctly arranged carboxyls. For this reason, only 2,4-PDCA is expected to affect both crucial events in connective tissue formation: designed as a specific inhibitor of prolyl 4-hydroxylase and therefore collagenous protein secretion [16,32], it is also predicted to be an inhibitor of the postulated PQQ ligase, interfering with the biosynthesis of functional lysyl oxidase and therefore with cross-link-stabilized matrix accumulation. In either case, the mode of action of 2,4-PDCA is reversible and competitive to each enzyme's co-substrate, i.e. 2-oxoglutarate or PQQ.

In contrast to 2,4-PDCA, which is not a known product of mammalian intermediary metabolism, both 3,4-DHPA and 3,4-DHMA are physiologically occurring compounds generated in the catabolic pathways of dopamine [35] and of norepinephrine/epinephrine [36], respectively. Humans synthesize the catecholamine metabolites 3,4-DHPA and 3,4-DHMA in milligram amounts per day [36,37]. The catecholamines are known to exert vital effects in the cardiovascular system [38], the same system that has elastin as a dominant structural protein [39]. The finding that 3,4-DHPA and 3,4-DHMA are potent ascorbate antagonists for prolyl 4-hydroxylase [19] and the reports on enhanced cross-link-stabilized elastin accumulation in the matrix of ascorbate-deprived, i.e. prolyl 4-hydroxylase-suppressed cell cultures [5–7], suggest that 3,4-DHPA and 3,4-DHMA may not be biologically inert degradation products: both have the potential to produce physiologically a relative ascorbate deficiency exclusively within the cisternae of the RER of susceptible cells, thus damping fibrogenesis via reduced secretion of hydroxyproline-rich triple-helical collagen, while boosting elastogenesis via secretion of hydroxyproline-deficient coacervate-prone elastin. In this way, the catecholamine metabolites 3,4-DHPA and 3,4-DHMA could biochemically contribute to the functionally required structure of vessels which bear the brunt of the catecholamine's biophysical effects. At least the generation of 3,4-DHPA from dopamine is in part dependent on a quinoprotein [44].

Considering the structure-activity relationship predicted and later verified for prolyl 4-hydroxylase antagonists [16–19,32] and the structure-activity relationship predicted here for the PQQ ligase, formulas for compounds can be suggested that may act as catalytic inhibitors of prolyl 4-hydroxylase *and* as biosynthetic inhibitors of lysyl oxidase. As shown in fig.3, omission of the *ortho* quinone moiety of PQQ and bioisosteric exchange of -NH- for $-CH_2-$ gives structure I whose arrangement of carboxyl groups is unaffected. Although prolyl 4-hydroxylase is inhibited by pyridine-2,5-dicarboxylate [17], indicating that the presence of a carboxyl function at the pyridine's C5 is well tolerated; the rigid bulky ring at C5 of structure I may interfere with effective attachment at the co-substrate-binding site. In structure II (fig.3) it was therefore substituted with a flexible aliphatic residue; its length may antagonistically affect the inhibitor's efficiency towards the hydroxylase and the ligase. As the subcellular target site – the cisternae of the RER/Golgi system – is shielded by two lipid barriers, the cytoplasmic and compartmental membranes, access of hydrophilic compounds like I and II is effectively blocked in vivo. Lipophilic bioactivatable pro-inhibitors, however, can be expected to reduce essentially the concentrations required for suppression of the intracisternal enzymes. In tissue culture and in the experimental animal, this approach was successfully used for 2,4-PDCA-mediated fibrosuppression [40,45]. The strategy holds the potential of restricting the intended effect to cer-

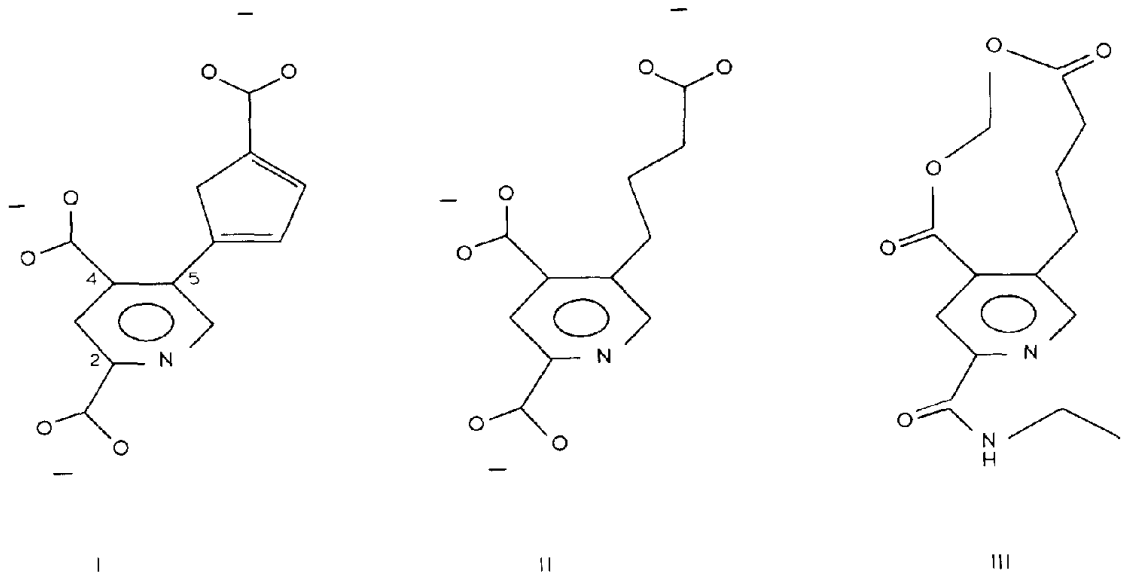

Fig.3. Design of structures potentially inhibitory both for prolyl 4-hydroxylase and for PQQ ligase.

tain organs as well, as an effective pro-inhibitor–inhibitor conversion has to occur intracellularly, and the pro-inhibitor activation can be designed to depend upon the metabolic peculiarities of the tissue at which the inhibitor's activity is aimed. Structure III of fig.3 exclusively serves to exemplify the theoretical combination of two different modifications reversibly masking the inhibitor's crucial carboxyl arrangement: amide and acetal formation. Biotransformation of an unpolar inactive pro-inhibitor like III, or its triethylamide analogue, to the polar active inhibitor is anticipated to occur preferentially in the portal system [41], regionally suppressing both peptidyl hydroxyproline formation and cross-linked-stabilized matrix accumulation – two highly desirable effects in any preventive or curative treatment of fibrotic liver diseases. In contrast, scarring processes compromising the function of elastic tissues, e.g. lung fibrosis, may respond to selective inhibition of hydroxyprolyl formation by 3,4-DHPA-derived pro-inhibitors that should not compromise elastogenesis.

For compounds of both the 2,4-PDCA and 3,4-DHPA class, pro-inhibitor targeting by specific chemical modifications and selective routes of administration to modulate connective tissue formation locally can be conceptualized that will prevent detrimental matrix alterations in distinct organs which otherwise disrupt their physiological function.

The demonstration that the reversible prolyl 4-hydroxylase inhibitors and the prosthetic group of lysyl oxidase share unique structural features is indicative of a common modulating mechanism that, at a given rate of protein biosynthesis, links post-translational events occurring 'upstream' and 'downstream' in extracellular matrix biosynthesis. The homology between 3,4-DHPA/3,4-DHMA/2,4-PDCA and PQQ holds biochemical implications relevant to the physiology and pathology of connective tissue formation and suggests that fibrogenesis and elastogenesis are differentially amenable to pharmacological therapeutics.

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