Pyridinedicarboxylates, the first mechanism-derived inhibitors for prolyl 4-hydroxylase, selectively suppress cellular hydroxyprolyl biosynthesis

Decrease in interstitial collagen and C1q secretion in cell culture

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Two pyridinedicarboxylates, predicted [Hansuske-Abel (1983) M.D.-Ph.D. Thesis, Philipps Universitäten Marburg] and later found to be potent reversible inhibitors of purified prolyl 4-hydroxylase [Majama, Hansuske-Abel, Günzler & Kivirikko (1984) Eur. J. Biochem. 138, 239-245] were investigated with respect to their effect on hydroxyprolyl biosynthesis in the fibroblast/collagen and the macrophage/C1q systems, and the effect was compared with that of the iron chelator 2,2’-dipyridyl, the compound used to inhibit cellular hydroxyprolyl formation. Only the enzyme-mechanism-derived pyridinedicarboxylates were highly selective inhibitors, and only they lacked overt cytotoxicity. Morphologically, their effect was restricted to the site of cellular hydroxyprolyl biosynthesis, i.e. the cisternae of the rough-surfaced endoplasmic reticulum. They were equally effective in the different cell types studied, and human and guinea-pig fibroblasts showed the same sensitivity. The minimal lipophilicity of the pyridinedicarboxylates necessitated high concentrations to achieve suppression of cellular hydroxyprolyl formation, but lipophilic bio-activatable pro-inhibitors may overcome this disadvantage. For the first time, experimental evidence is presented suggesting that, in cell culture, the biosynthesis of interstitial collagens and C1q can be suppressed selectively, identifying the pyridinedicarboxylates as promising pilot compounds for experiments in vivo.

INTRODUCTION

Prolyl 4-hydroxylase (procollagen-proline, 2-oxoglutarate 4-dioxygenase, EC 1.14.11.2) catalyses one of the crucial intracellular post-translational modifications in the biosynthesis of collagenous proteins, the stereospecific hydroxylation of certain primary-structure-defined proline residues. The αβγδ-tetrameric enzyme, a non-haem-iron dioxygenase exclusively located in the cisternae of the rough-surfaced endoplasmic reticulum, utilizes molecular O₂ and 2-oxoglutarate as co-substrates, and non-stoichiometrically requires ascorbate under physiological conditions [1]. trans-4-Hydroxyproline, at 37 °C, is indispensable for the formation and the stability of the collagen triple helix, and collagenous molecules deficient in hydroxyprolyl do not display this helical structure [2]. In vitro, interference with the enzyme’s activity by non-specific inhibitors such as iron chelators or proline analogues suppresses the secretion of interstitial collagens [2] and C1q [3–5]. This finding led to the suggestion that cells control the conformation of collagenous proteins before secretion, and retain non-functional non-triple helical molecules [2]. For these reasons, prolyl 4-hydroxylase became one of the target enzymes in the quest for scientifically and, in the end, clinically applicable fibro suppressive and immune-suppressive agents [6,7].

To provide a rational basis for the receptor-guided development of such specific inhibitors, a detailed stereochemical concept for the catalytic mechanism of this enzyme was published by Hansuske-Abel & Günzler [6]. The complete sequence of this ‘HAG’ mechanism should consist of two half-reactions: the decarboxylation of 2-oxoglutarate with concomitant ferryl generation, and the consecutive ferryl-mediated hydroxylation of prolyl via a radical-abstraction–recombination step. According to the ‘HAG’ mechanism, the enzyme’s 2-oxoglutarate-binding site, i.e. the target receptor, should display subsites for salt-bridge formation with the C-5 carboxy group of the co-substrate, and for chelation of its α-oxo acid moiety. That subsite, the enzyme-bound iron atom, was suggested to mediate decarboxylation and ferryl formation in a ligand reaction characterized by a tetrahedral transition state of the C-2 of 2-oxoglutarate [6]. This structural and functional analysis of the target receptor allowed the knowledge-based design of several classes of co-substrate antagonists able to attach to the 2-oxoglutarate-binding site of enzyme, but unable to participate in the ferryl-generating ligand reaction, in this way interfering with the first phase of the catalytic cycle [6,8].

Pyridine-2,4-dicarboxylate (2,4-PDCA) was predicted to act in this way, reversibly blocking the target receptor and competitively suppressing 2-oxoglutarate utilization for ferryl formation [8]. Indeed, this compound and its isomer pyridine-2,5-dicarboxylate (2,5-PDCA) were immediately identified as potent inhibitors of prolyl hydroxylase [9]. The pyridinedicarboxylates provided detailed insight into the electronic and steric prerequisites for this enzyme. The present study attempts to provide a stereochemical rationale for the inhibition of the enzyme, and thus to gain a deeper understanding of the pyridinedicarboxylates’ potential as a tool for the development of new fibro suppressive agents.

Abbreviations used: 2,4-PDCA and 2,5-PDCA, pyridine-2,4-dicarboxylate and pyridine-2,5-dicarboxylate respectively.
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for the decarboxylation step, i.e. the ferryl generation, and allowed the mapping of the 2-oxoglutarate-binding site of both prolyl 4-hydroxylase and lysyl hydroxylase [9,10]. 2,4-PDCA was successfully employed as a probe to study stoichiometry and kinetics of the enzyme’s half-reaction [11]. The 5-azido derivative of 2,5-PDCA, used as an affinity label for the 2-oxoglutarate-binding site, covalently bound to the \( \alpha \)-subunits only, providing the first experimental evidence for the subunit location of the active site, [12]. Being competitive with respect to 2-oxoglutarate and non-competitive with respect to iron, and displaying a \( K_{f} \) in the region of 1 \( \mu \)M, 2,4-PDCA and 2,5-PDCA were the first well characterized, potent and specific inhibitors of the purified collagen hydroxylases. Other 2-oxoglutarate-metabolizing enzymes, e.g. the 2-oxoglutarate dehydrogenase system of the tricarboxylic acid cycle, are insensitive to these compounds [10].

The finding that the pyridinedicarboxylates select for the 2-oxoglutarate-binding site of collagen hydroxylases invited an investigation of their effect on prolyl 4-hydroxylase-modified proteins in cell culture. In the present paper we report that the pyridinedicarboxylates selectively suppress cellular hydroxyprolyl biosynthesis. Irrespective of the cellular systems scrutinized, 2,4-PDCA is more efficient than 2,5-PDCA, contrasting with the findings of the enzyme studies [9]. Moreover, the biosynthesis of structurally very dissimilar collagenous proteins from different species and different tissues is equally susceptible to these inhibitors.

**MATERIALS AND METHODS**

**Materials**

2,4-PDCA, 2,5-PDCA and 2,2'-bipyridyl were purchased from Aldrich, Steinheim, Germany. Kits for determination of lactate dehydrogenase came from Merck, Darmstadt, Germany. [U-\(^{14}\)C]Proline was from New England Nuclear, Dreieich, Germany, and [4,5-\(^{3}H\)]leucine was from Amersham, Braunschweig, Germany. Media were ordered from Biochrom, West Berlin, Germany. All other chemicals were from Sigma Chemie, Deisenhofen, Germany. Fibronectin was purified by the procedure of Ruoslahti et al. [13].

**Fibroblast cultures**

Locally established human and guinea-pig skin fibroblast cultures in the seventh passage were grown to confluence. Previously, the type I/type III collagen ratio in medium was electrophoretically determined to be 10:1 and 10:0.7 respectively; as revealed by collagenase digestion [14] of medium, collagens represented 4% of secreted proteins of fibroblasts from both species. After preincubation for 24 h without serum in glutamine-free Dulbecco's minimal essential medium containing 50 \( \mu \)g of sodium ascorbate, 60 \( \mu \)g of 3-aminopropionitrile and 100 units of penicillin G per ml, the cultures were exposed for 20 min to the individual agents in appropriate concentration, followed by the addition of 2 \( \mu \)Ci of [U-\(^{14}\)C]proline or [4,5-\(^{3}H\)]leucine per ml for 5 h. The medium was removed on ice, and the cell layer was washed three times with ice-cold phosphate-buffered saline (0.14 M-NaCl/0.96 mm-sodium phosphate buffer), pH 7.5, and harvested with a rubber ‘policeman’.

**Macrophage cultures**

Starch-elicited peritoneal macrophages of guinea pigs were harvested in medium M199 as described previously [3,5]. The protocol for preincubation, for metabolic labelling and for processing of cell layer and medium was identical with that of fibroblasts. Electrophoretically, no collagen-\( \alpha \)-chain-like material could be demonstrated after metabolic labelling.

**Determination of compound toxicity**

As a sensitive indicator for damage to the cytoplasmic membrane and for cell death [15], lactate dehydrogenase activity was determined in samples of centrifuged medium and sonicated cells of each flask. In addition, Eosin dye exclusion was used to check viability of treated and untreated monolayers. General protein biosynthesis was evaluated by determining the total incorporation of [4,5-\(^{3}H\)]leucine into protein precipitated at 4 °C in the presence of 10% (w/v) trichloroacetic acid and 2 mm-leucine, and washed three times with 5% (w/v) trichloroacetic acid/2 mm-leucine.

**Determination of radioactive leucine, proline and hydroxyproline in trichloroacetic acid-precipitable protein**

All subsequent steps were carried out at 4 °C. Cellular protein was precipitated by mixing with a solution of trichloroacetic acid, containing unlabelled leucine or proline, to give a final concentration of 10% (w/v) trichloroacetic acid and 2 mm of the respective amino acid. To the medium, bovine serum albumin was added as carrier in a final concentration of 50 \( \mu \)g/ml. The precipitate was collected by centrifugation (1500 g for 5 min) and washed three times with a solution containing 5% trichloroacetic acid and 2 mm-leucine or -proline. This final precipitate was hydrolysed in 6 M-HCl at 110 °C for 16 h. After acid removal, the samples were applied to a BioTronic automated amino acid analyser running a routine program without ninhydrin reagent, and equipped with a fraction collector to sample the eluent. Radioactivity was determined, after addition of 10 ml of Biofluor (New England Nuclear) per ml of eluent, in a Kontron liquid-scintillation counter.

**Electrophoretic studies**

The final precipitate was also used for SDS/polyacrylamide-gel electrophoresis under standard conditions [16]. The samples, reduced by 5% (v/v) 2-mercaptoethanol, were run routinely in a Pharmacia system on gels with a linear 5–10% gradient of polyacrylamide, and subsequently processed for fluorography [17]. The bands were quantified by scanning at 540 nm with a computerized Hirschmann Elscript 2000 densitometer. For the investigation of collagen biosynthesis, densitometric data derived coefficients were found to provide valuable information [18].

**Electron-microscopic studies**

Monolayers of fibroblasts, unexposed and exposed for 5 h to 3 mm-2,4-PDCA, were fixed on ice in 0.1% glutaraldehyde/0.15 M-cacodylate buffer, pH 7.4, for 45 min, passed through 0.5% OsO\(_4\) in 0.1 M-phosphate buffer, pH 7.0, dehydrated and embedded in Epon resin. Thin sections, cut with a Siemens microtome diamond knife, were picked up on Formvar-coated grids, counterstained with 0.7% uranyl acetate in 70% (v/v) ethanol and 0.8% lead citrate [19] and examined in a Philips 301 electron microscope.
Table 1. Dose-response relationship of compounds in cultured human skin fibroblasts, determined by metabolic labelling as specified in the Materials and methods section

Values are the means for three experiments. Overall protein biosynthesis was measured by [3H]leucine incorporation, total hydroxyprolyl formation by synthesis of hydroxy[14C]prolyl from [14C]proline. The findings obtained in guinea-pig fibroblast cultures were virtually identical (results not included). Suppression of total cellular hydroxyprolyl formation required concentrations of the hydrophilic pyridinedicarboxylates significantly above their $K_i$ values, whereas the corresponding concentrations of the lipophilic 2,2'-bipyridyl were in a similar range. Only the pyridinedicarboxylates, however, decreased total hydroxyprolyl formation without significantly affecting overall protein biosynthesis. Abbreviation: N.D., not determined.

<table>
<thead>
<tr>
<th>Conc. required for indicated inhibition (mM)</th>
<th>2,4-PDCA ($K_i = 2 \mu M$)</th>
<th>2,5-PDCA ($K_i = 0.8 \mu M$)</th>
<th>2,2'-Bipyridyl ($K_{app} = 10 \mu M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% = ID$_{50}$</td>
<td>1</td>
<td>2.7</td>
<td>0.01</td>
</tr>
<tr>
<td>50% = ID$_{50}$</td>
<td>2.2</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>90% = ID$_{90}$</td>
<td>4.5</td>
<td>$&gt; 8$</td>
<td>0.10</td>
</tr>
<tr>
<td>Relative decrease in overall protein biosynthesis (%)</td>
<td>2,4-PDCA</td>
<td>2,5-PDCA</td>
<td>2,2'-Bipyridyl</td>
</tr>
<tr>
<td>ID$_{50}$</td>
<td>2</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>ID$_{50}$</td>
<td>6</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>ID$_{90}$</td>
<td>14</td>
<td>N.D.</td>
<td>80</td>
</tr>
</tbody>
</table>

RESULTS

Toxicity of compounds

Eosin dye exclusion, release of cytoplasmic lactate dehydrogenase and measurement of total protein biosynthesis, as determined by incorporation of [U-14C]-proline or [4,5-3H]leucine, agreed in revealing the marked toxicity of 2,2'-bipyridyl and the low toxicity of the pyridinedicarboxylates at the inhibitory doses that suppressed cellular hydroxyprolyl biosynthesis by 50, 75 and 90%, i.e. at the ID$_{50}$, ID$_{75}$ and ID$_{90}$. With all compounds the dose-dependent increase of lactate dehydrogenase release, indicating cellular damage, was closely linked to the decline of [4,5-3H]leucine incorporation irrespective of the cellular system studied (results not shown). 2,2'-Bipyridyl produced striking enhancements of lactate dehydrogenase release and a concomitant decrease in leucine incorporation, which paralleled the decrease in hydroxyprolyl biosynthesis as shown in Table 1 and Figs. 1 and 2. In contrast, the pyridinedicarboxylates, even at concentrations that maximally inhibited cellular hydroxyprolyl generation, increased lactate dehydrogenase release and decreased total protein biosynthesis only marginally. At the ID$_{50}$ of 2,4-PDCA for hydroxyprolyl generation in both the fibroblast and the macrophage systems, the effect on Eosin dye exclusion, lactate dehydrogenase release and total protein biosynthesis was within the limits of experimental error.

Dose-dependency of effects

All substances tested gave a concentration-dependent decrease in cellular hydroxyprolyl generation during the labelling period, which previously was found to ensure linear incorporation of amino acids into protein synthesized de novo. Essentially, the dose–response curve for each compound did not change with the hydroxyprolyl-generating cell type studied. Moreover, the effect of the compounds on human fibroblasts, as summarized in Table 1, was similar to that on guinea-pig fibroblasts, with no observable species difference. It was noted earlier that the significant difference in the ID$_{50}$ of the pyridinedicarboxylates and of 2,2'-bipyridyl (Table 1) correlates with the lipophilicity of the compounds: the distribution coefficient in the biphasic system octanol/phosphate-buffered saline, pH 7.4, is 50 for 2,2'-bipyridyl, but only 0.003 for the pyridinedicarboxylates [20]. In both hydroxyprolyl-producing cell types from the guinea pig, 2,4-PDCA was the more dose-efficient inhibitor than 2,5-PDCA (cf. Figs. 1 and 2). This observation was corroborated for the hydroxyprolyl generation by human fibroblasts: at the ID$_{50}$ of 2,4-PDCA 2,5-PDCA displays its ID$_{50}$ (Table 1).

Effect of compounds in guinea-pig and human skin fibroblast cultures

Total hydroxyprolyl biosynthesis in fibroblasts of these two species was equally susceptible to the inhibitory effect of the pyridinedicarboxylates, and no inter-species difference in potency could be demonstrated (cf. Table 1). The ID$_{50}$ was 5 mM for 2,5-PDCA and about 2 mM for 2,4-PDCA. At this concentration 2,4-PDCA essentially did not diminish total protein biosynthesis, and even at its ID$_{50}$ had less effect on this parameter than 2,5-PDCA at its ID$_{50}$. Although at 5.5 mM-2, 4-PDCA hydroxyprolyl generation by human skin fibroblasts was decreased by 95%, total protein biosynthesis still was 80% of the controls. 2,4-PDCA gave this selective suppression of cellular hydroxyprolyl synthesis in both species. By contrast, 2,2'-bipyridyl diminished this parameter to the same degree as total protein biosynthesis, and a preferential effect on hydroxyprolyl generation could not be established in the present study (cf. Table 1).

The differential determination of hydroxyprolyl in
Fig. 1. Extracellular effect of compounds in cultures of guinea-pig macrophages

In the presence of the indicated inhibitor concentrations, secretion of protein synthesized de novo was determined as [3H]leucine content of trichloroacetic acid-precipitable macromolecules of the medium, and secreted peptidyl hydroxyproline as [14C]-proline-derived hydroxy[14C]proline in the same samples. ■, [3H]leucine content; ○, hydroxy[14C]proline content. (a) 2,4-PDCA; (b) 2,5-PDCA; (c) 2,2'-bipyridyl.

Fig. 2. Intracellular effect of compounds in cultures of guinea-pig macrophages

In the presence of the indicated inhibitor concentrations, protein synthesized de novo was determined as [3H]leucine content of trichloroacetic acid-precipitable macromolecules in the cell layer, and peptidyl hydroxyproline as [14C]proline-derived hydroxy-[14C]proline in the same samples. ■, [3H]leucine content; ○, hydroxy[14C]proline content. (a) 2,4-PDCA; (b) 2,5-PDCA; (c) 2,2'-bipyridyl.
Table 2. Indicators, in human skin fibroblast cultures, of the compounds' activity with respect to intracellular retention of interstitial collagens, and to release of non-collagenous compared with collagenous connective-tissue proteins

Relative retention and relative release were calculated from the indicated densitometry-derived coefficients for accumulation and secretion by arbitrarily assigning '1.0' to the controls and using this value as numeric reference. Only the pyridinedicarboxylates produced a preferential decrease in collagen secretion, with significant intracellular accumulation of collagenous material.

<table>
<thead>
<tr>
<th></th>
<th>Accumulation coefficient*</th>
<th>Relative retention</th>
<th>Secretion coefficient†</th>
<th>Relative release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.21</td>
<td>1.0</td>
<td>0.14</td>
<td>1.0</td>
</tr>
<tr>
<td>2,2'-Bipyridyl (ID$_{75}$ = 100 µM)</td>
<td>1.18</td>
<td>5.61</td>
<td>0.12</td>
<td>0.85</td>
</tr>
<tr>
<td>2,4-PDCA (ID$_{75}$ = 3 mM)</td>
<td>7.9</td>
<td>37.62</td>
<td>1.21</td>
<td>8.64</td>
</tr>
<tr>
<td>2,5-PDCA (ID$_{75}$ = 7 mM)</td>
<td>1.9</td>
<td>9.04</td>
<td>0.84</td>
<td>6.00</td>
</tr>
</tbody>
</table>

* Calculated from the densitometric analyses of the fluorograms depicting $^{14}$C-labelled protein as:

**Intracellular [pro $\alpha$]**

Extracellular [pro $\alpha + pC \; \alpha + pN \; \alpha + \alpha$]

where pro $\alpha$ represents procollagen $\alpha$-chains, p$C \; \alpha$ and p$N \; \alpha$ represent C-terminal and N-terminal fragments of collagen $\alpha$-chains, and $\alpha$ represents collagen $\alpha$-chains.

† Calculated from the densitometric analyses of the fluorograms depicting $^{14}$C-labelled protein as:

**Extracellular [fibronectin]**

Extracellular [pro $\alpha + pC \; \alpha + pN \; \alpha + \alpha$]

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Fig. 3. Electron micrograph of fibroblast, control (magnification × 4500)

The rough-surfaced endoplasmic reticulum appears as tender network or parallel membranes.

trichloroacetic acid-precipitable proteins from cells and from medium revealed a relative intracellular enrichment of hydroxyprolyl-containing material coincident to the increase of inhibitor concentration (results not shown). In collagen-producing cells this finding is known to be a characteristic consequence of lowered prolyl hydroxylase activity [2]. Intracellular retention of procollagen chains was also suggested by the densitometric analysis of fluorograms depicting the metabolically labelled proteins from cells and from medium in the absence of inhibitors and at their ID$_{75}$ concentrations. Although 2,2'-bipyridyl produced a 5-fold and 2,5-PDCA a 9-fold increase in intracellular compared with extracellular $\alpha$-chains, the relative retention caused by 2,4-PDCA was almost 40 times that of the control (Table 2). When compared with collagenous material, the release of the non-collagenous
fibronectin was not decreased by the pyridinedicarboxylates, but was decreased by 2,2'-bipyridyl (Table 2). This finding attests to the pyridinedicarboxylate's ability to diminish selectively the secretion of hydroxyprolyl-dependent proteins without affecting the extrusion of hydroxyprolyl-independent proteins.

The electron-microscopic pictures revealed the morphology of this effect: human fibroblasts, incubated with 2,4-PDCA at its ID50 for 5 h, showed no overt signs of morphological damage, the mitochondria appeared to be unaffected and pinocytotic processes at the cell membrane were undisturbed; however, in contrast with the controls, the cisternae of the rough-surfaced endoplasmic reticulum were bulging and loaded with an amorphous material (cf. Figs. 3 and 4) that reacted with monospecific anti-(human type I collagen) antibodies (M. Ragunath, G. Tschank, H.-J. Rumpelt, K. Becker & H. M. Hanauske-Abel, unpublished work). Identical results were obtained for 2,5-PDCA (results not shown). Thus only that cellular compartment which exclusively harbours prolyl hydroxylase and its substrate [2] is altered by the pyridinedicarboxylates.

Effects of compounds in guinea-pig macrophage cultures

All the compounds tested suppressed total hydroxyprolyl generation by these cells, each with a dose-response relationship virtually identical with that obtained in the fibroblast cultures. The pyridinedicarboxylates showed a preferential decrease in total hydroxyprolyl generation, whereas 2,2'-bipyridyl diminished this parameter only to the same extent as total protein biosynthesis. The differential determination of label in proteins from medium and cells revealed that 2,4-PDCA, without significant effects on biosynthesis and extrusion of hydroxyprolyl-free proteins, almost completely abolished the presence of extracellular hydroxyprolyl-containing peptides at 5.5 mM. At this concentration the 2,5-PDCA-mediated suppression of hydroxyprolyl in the trichloroacetic acid-precipitable material from medium was not lower than 30%, with overall protein secretion still 90% of the controls, however (Fig. 1). In contrast with the pyridinedicarboxylates, 2,2'-bipyridyl decreased the hydroxyprolyl content in intracellular and extracellular peptides in parallel with both total protein biosynthesis and overall protein secretion (cf. Figs. 1 and 2). The relative retention, i.e. the ratio of extracellular to intracellular peptidyl hydroxyproline, indicates that an intracellular accumulation of hydroxyprolyl-containing material occurred. At the highest concentration of each compound tested, relative retention for 2,4-PDCA is 9.6, for 2,5-PDCA 1.3, and for 2,2'-bipyridyl 2.5. However, the relative release, i.e. the ratio of extracellular peptidyl...
leucine to extracellular peptidyl hydroxyproline, which reflects the preference for suppressing secretion of molecules with collagen-like structure, is strikingly divergent: whereas the value for 2,2'-bipyridyl is 0.35, those for 2,4-PDCA and 2,5-PDCA are 37.4 and 2.8 respectively. Thus, relative to collagenous material, the release of non-collagenous protein was not decreased by the pyridinedicarboxylates, but was decreased by 2,2'-bipyridyl.

DISCUSSION

The pyridinedicarboxylates studied here, predicted to interfere with the decarboxylation phase of the catalytic cycle of prolyl 4-hydroxylase [8], were recently recognized as the most potent reversible inhibitors presently available for this purified enzyme: their $K_i$ values are in the region of 1 $\mu$M and they are, in accordance with their anticipated action, competitive with respect to 2-oxoglutarate, the substrate of the enzyme's initial decarboxylation phase [9,10]. We have now investigated the effect of this class of prolyl 4-hydroxylase inhibitors on cellular hydroxyprolyl biosynthesis, using cells of different species that synthesize one class of collagenous proteins, and cells of one species that synthesize different classes of collagenous proteins. That strategy allowed us to investigate, in addition to the general effects of the pyridinedicarboxylates in culture, their potentially differential effects on collagens and C1q, structurally very dissimilar proteins that nevertheless are hydroxyprolyl-dependent with respect to their function and biosynthesis [2–5]. 2,2'-Bipyridyl was selected as the reference compound for two reasons: firstly, the pyridinedicarboxylates' structures identify them as hybrid molecules of 2,2'-bipyridyl and 2-oxoglutarate (Fig. 5); secondly, 2,2'-bipyridyl initially was used to show the hydroxyprolyl-dependent secretion of interstitial collagens and of C1q [2,3] and to demonstrate the pivotal role of prolyl 4-hydroxylase activity for C1q functionality [3–5].

In the fibroblast and macrophage cultures, 2,2'-bipyridyl at concentrations that inhibited hydroxyprolyl formation was found to be a highly toxic compound, reaffirming earlier observations, e.g. the striking decrease in total protein biosynthesis and overall protein secretion caused by this compound [21]. Under the same conditions, no significant toxicity of the pyridinedicarboxylates could be demonstrated.

The insufficient specificity of 2,2'-bipyridyl, which in contrast to the pyridinedicarboxylates interacts with iron ions to give kinetically stable low-spin complexes [22], was emphasized previously [2]. The data presented here also do not suggest any appreciable selectivity: total hydroxyprolyl biosynthesis declined in parallel with overall protein biosynthesis (Table 1 and Figs. 1 and 2), and secretion of hydroxyprolyl-containing protein in parallel with protein secretion in general (Fig. 1). In contrast, the effect of the pyridinedicarboxylates on these parameters was strikingly differential: hydroxyprolyl biosynthesis and secretion of hydroxyprolyl-containing protein were affected selectively (Table 1 and Figs. 1 and 2). This observation coincides with the densitometric analysis of collagenous and non-collagenous proteins synthesized and secreted by human skin fibroblasts in the presence of the test compounds. 2,2'-Bipyridyl decreased the extracellular amount of fibronectin relative to that of collagen, and the pyridinedicarboxylates significantly decreased the amount of collagen relative to that of fibronectin (Table 2).

Morphologically, the pyridinedicarboxylates exclusively affected the cisternal space, the site of hydroxyprolyl formation within specific peptide substrates. This observation (Figs. 3 and 4), the intracellular/extracellular distribution of collagen $\alpha$-chains in fibroblast cultures (Table 2), as well as the corresponding determination of hydroxyprolyl in fibroblasts (results not shown) and macrophage cultures (Figs. 1 and 2), indicate a retention of underhydroxylated peptide substrate in the cisterna of the rough-surfaced endoplasmic reticulum. This effect did not result in demonstrable cell damage, however, suggesting the presence of mechanisms that curtail intracisternal accumulation of the peptide substrates for prolyl 4-hydroxylase, most probably by restricting translation/transcription of their m-RNAs. Collagen peptides have been suggested to act as translational and pretranslational regulators of collagen biosynthesis [23]. In conclusion, selective suppression of prolyl 4-hydroxylase by 2,4-PDCA may produce secondary inhibitory effects in the biosynthetic pathway of hydroxyprolyl-dependent proteins. Searching for potential additional effects resulting directly from the structure of that molecule, we observed that, although originally an inhibitory pilot compound deduced from the orbital intersections during the first catalytic step mediated by the intracellular prolyl 4-hydroxylase [6,8], 2,4-PDCA electronically and structurally mimics an essential part of pyrroloquinoline quinone [24], the natural compound that is the cofactor of the extracellular lysyl oxidase [25, 26]. Both enzymes mediate key post-translational modifications in collagenous proteins and in elastin. It was suggested that pyrroloquinoline quinone-like
molecules function as physiological modulators of matrix protein biosynthesis [24].

The finding that the pyridinedicarboxylates do not possess significant cytotoxic characteristics although they are structural analogues of 2-oxoglutarate, a key molecule of the citric acid cycle and numerous catabolic and anabolic pathways, reflects the singularity of the 2-oxoglutarate-binding site of prolyl 4-hydroxylase, which necessitates chelation to catalytic-centre Fe\(^{3+}\) ion for effective interaction, as shown in Fig. 5 [6]. 2-Oxoglutarate dehydrogenase, the citric acid-cycle enzyme that like prolyl 4-hydroxylase catalyses 2-oxoglutarate decarboxylation, but does not require an active-site Fe\(^{3+}\) ion, is insensitive to the pyridinedicarboxylates [10], indicating, like the cytotoxicity findings reported in the present paper, that these compounds are not indiscriminate 2-oxoglutarate antagonists.

The data presented argue against a species-specific action of the pyridinedicarboxylates, as cultured human and guinea-pig fibroblasts did not show appreciable differences in sensitivity. To study potential cell-specific actions, the hydroxyproply formation in the macrophage/Clq system was compared with that in the fibroblast/collagen system. In contrast with fibroblasts, macrophages are known to possess prolyl 4-hydroxylase activity in large excess of hydroxylatable peptide substrate [27]. For this reason, it was suggested that Clq biosynthesis by these cells should be insensitive to specific prolyl 4-hydroxylase inhibition [28]. However, the ID\(_{50}\) for pyridinedicarboxylate-mediated suppression of hydroxyproply formation in the macrophage/Clq system is not significantly different from that in the fibroblast/collagen system. Therefore, these results do not point to distinct prolyl 4-hydroxylase activities or isoenzymes in matrix-generating and in immunocompetent cells.

Although Clq functionality was not directly determined in the present study, inhibition of prolyl 4-hydroxylase in the macrophage system used here has previously been shown to affect strikingly the haemolytic activity of Clq [3–5]. For this reason it may be concluded that the pyridinedicarboxylate-induced decrease in hydroxyproply formation in the macrophage cultures, which were shown electrophoretically to lack collagen \(\alpha\)-chain synthesis, coincided with a loss of Clq functionality.

In culture, the target enzyme is shielded by the cell membrane, the cytoplasm and the membrane of the rough-surfaced endoplasmic reticulum. It was anticipated that the restrictions imparted by these structures, such as permeability, protein binding or metabolic inactivation, were crucial for the development of prolyl 4-hydroxylase inhibitors [6]. The results obtained indeed reflect the decisive role of these structures, especially the membranes. In studies of the purified enzyme, the \(K_c\) for 2,5-PDCA was about one-third that of 2,4-PDCA [9], whereas in culture, irrespective of cell type or species, the ID\(_{50}\) for 2,5-PDCA was at least twice that of 2,4-PDCA. Thus, going from hydroxyproply formation \(\text{in vitro}\) to that \(\text{in cellula}\), the effectiveness of the pyridinedicarboxylates becomes inverted. Moreover, their concentrations required to inhibit 50% of each mode of hydroxyproply formation differ by a factor of 1000-fold: e.g. 2,4-PDCA displays a \(K_c\) of about 2 \(\mu\)m, but an ID\(_{50}\) in the region of 2 mm. 2,2'-Bipyridyl does not display such a test-system-dependent gap of dose effectiveness, with identical iron concentrations provided [20,22]. This observation is, at least in part, attributable to the different lipophilicity of the compounds: whereas the non-polar 2,2'-bipyridyl can easily penetrate the two lipid bilayers shielding the enzyme, the diatomic pyridinedicarboxylates cannot do so [20].

Although the substance had to be applied in millimolar concentrations, the results of our study suggest that at least 2,4-PDCA is the first promising pilot compound for selective interference with cellular hydroxyproply generation. The structure lends itself to modifications that produce a bioactivatable lipophlic pro-inhibitor. Recently, we found that micromolar concentrations of the diethyl ester of this compound selectively suppresses collagen biosynthesis in chicken calvaria (G. Tschan, D. Brocks, J. Mohr, K. Engelbart, V. Günzler & H. M. Hanauske-Abel, unpublished work). This pro-inhibitor strategy holds the promise of targeting a prolyl 4-hydroxylase inhibitor to only those tissues and cells that are able to mediate the pro-inhibitor–inhibitor conversion, in this way achieving organ-specificity of a fibrosuppressive regimen.

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