Journal of Medicinal Chemistry

Volume 24, Number 6 June 1981

Perspective

Perspectives for the Use of Collagen Synthesis Inhibitors as Antifibrotic Agents

George C. Fuller

Searle Research and Development, Division of G.D. Searle and Co., Chicago, Illinois 60680. *Received March 24, 1981*

Introduction

The fibrotic diseases are a family of acquired connective tissue diseases characterized by the overaccumulation of fibrous components in the extracellular matrix. Although the terminal fibrotic lesion in these diseases is uniformly considered to be a sequela of cellular injury, the cell populations injured and the endogenous mediators responsible for the postinjury fibrotic response vary from organ to organ. The end result of a fibrogenic response in a parenchymatous organ is distortion or destruction of the parenchymal architecture resulting in loss of function. In the cardiovascular system the fibrotic vascular architectural derangement results in the loss of vessel wall elasticity and surfaces that are compatible with platelets. Fibrosis in all of these situations usually remains undetected until it is well established and has progressed toward end-stage organ failure. These factors, combined with the slow turnover of matrix collagen, contributed to the prefactory conclusion that fibrosis was an untreatable and irreversible lesion. Recent demonstration projects using proline analogues or inhibitors of collagen cross-linking now indicate that this view is incorrect and that pharmacological control of fibrosis through inhibition of collagen synthesis or processing is an achievable objective. The importance of antifibrotic drug development is evident when one considers the high frequency of occurrence of the fibrotic lesion in Western society and the increasing death rate resulting from end-stage fibrosis of various organs (liver, lung, kidney, cardiovascular). These serious medical problems have been editorial topics in recent clinical literature.[1,2]

The extracellular matrix of the mammal is composed of two fibrous proteins, elastin and collagen, surrounded by proteoglycans. The architectural development and function of each organ is influenced by and dependent upon the heterogeneity of collagen and proteoglycan, as well as the relative ratio of all three matrix components. Accordingly, chemical derangement of the matrix components will result in altered structure and compromised function of the organ. Fibrosis usually occurs when the chemical derangement is an overaccumulation of collagen, but in some forms of lung fibrosis changes in the geometric distribution of collagen in the organ without overaccumulation may be responsible for the loss of function. There appears to be ample evidence that this fibrotic collagen accumulation is the result of overproduction as opposed to decreased degradation. However, it is not possible at the present time to exclude increases in the rate of maturation (the rate of extracellular cross-link formation) or changes in collagen heterogeneity as causative factors in the development of a fibrotic lesion in view of their probable influence on collagen turnover. The literature describing collagen synthesis, processing, and degradation and the role of these in the fibrotic process has been compiled and discussed in several recent reviews.[3-5]

Attempts to influence fibrotic connective tissue accumulation have focused on agents which alter the course of the inflammatory events preceding fibrosis and on agents which impair collagen synthesis or processing. This latter group includes inhibitors of matrix cross-link formation which have been studied extensively as antifibrotic agents. Penicillamine has been tested as a potential antifibrotic agent because of its ability to block reactive aldehydes and, therefore, prevent collagen cross-linking. β-Aminopropionitrile (BAPN), used in short-term clinical trials for the past decade, inhibits lysyl oxidase and, therefore, decreases collagen cross-linking. Animal studies have established that subtoxic doses of BAPN reduce collagen accumulation in a number of fibrosis models, including liver. In recent clinical studies, purified forms of BAPN have been administered in doses up to 1 g/day without acute hypersensitivity reactions. However, the prolonged use of BAPN to prevent collagen deposition in chronic fibrotic disease presents the risk of producing a lathyritic syndrome due to a requirement for an identical cross-linking reaction for the synthesis of elastin. This effect is viewed as a major liability to further development of BAPN as an antifibrotic agent for use in man.

The current interest in proline analogues as antifibrotic agents is based on their ability to (a) decrease collagen synthesis, (b) to result in peptides which inhibit prolyl hydroxylase, and (c) to result in the production of collagens which are not hydroxylated. Various proline analogues have been shown to prevent lung and liver fibrosis in animal models. Since these analogues are incorporated into

(1) Karlinsky, J. B.; Goldstein, R. H. *J. Lab. Clin. Med.* **1980**, *96*, 939–942.
(2) Bailey, A. J.; Duance, V. C. *Eur. J. Clin. Invest.* **1980**, *10*, 1–3.
(3) Prockop, D. J.; Kivirikko, K. K.; Tuderman, L.; Guzman, N. A. *N. Engl. J. Med.* **1979**, *301*, 13–23, 77–85.
(4) Fuller, G. C.; Mann, S. W. *Rev. Biochemical Toxicol.* **1981**, *3*, 193–230.
(5) Minor, R. R. *Am. J. Pathol.* **1980**, *98*, 226–278.

0022-2623/81/1824-0651$01.25/0

all proteins in place of proline, noncollagen protein synthesis is also inhibited and minor changes in the function and conformation of some proteins have been reported. The literature describing the pharmacology and toxicology of the existing antifibrotic agents has also been compiled and discussed in a recent review.[6] The experience gained through the use of these compounds in animal models of fibrosis and the success of a few of these as model compounds in limited clinical trials have provided documentation that control of collagen deposition in fibrotic organs is achievable and that this control can be used as a means of preventing end-stage fibrosis.

The limited search for drugs acting directly and specifically on the collagen biosynthetic pathway during the past decade has been largely attendant to the growth of interest in the molecular biology of matrix formation. As a result, attempts to control the fibrotic lesion have been restricted to the naturally occuring inhibitors described above (penicillamine, BAPN, and proline analogues). Since several potentially drug vulnerable sites have been identified and characterized in the collagen biosynthetic pathway, antifibrotic drug development should be carried forward on several fronts.

Overview of Collagen Matrix Synthesis and Metabolism

Because of the central role of the collagen pathway in the fibrotic process, it is necessary to describe the components of the pathway with respect to their potential as sites of drug action. The primary structures of the various collagen gene products and the molecular characteristics of the pathway have been studied extensively during the past 2 decades. Comprehensive reviews of this material are available.[7-9]

Collagen synthesis occurs in a series of sequential steps consisting of the assembly of proline-rich and lysine-rich polypeptide precursors of collagen (procollagen α chains), enzymatic hydroxylation of some of the prolyl and lysyl residues, and glycosylation of some of the hydroxylysyl residues. The cellular processing of the procollagen α chains requires the assembly of three procollagen chains to form a triple helix with stability provided by disulfide bonds in the nonhelical regions of the molecule. After the secretion of procollagen into the extracellular space, these nonhelical regions are removed by extracellular procollagen peptidases. The cross-linking of collagen occurs through further extracellular processing as a result of the enzyme lysyl oxidase, which oxidatively deaminates specific lysine and hydroxylysyl residues to leave aldehyde moieties that form cross-links through condensation reactions. The molecular packing of the processed helical domains results in a rigid structure composed of triple helical collagen molecules in a covalently bonded configuration that is resistant to most proteases. This fibril structure is identified by its characteristic periodicity when interstitial collagen is examined by electron microscopy or as amorphous basement membrane structures containing type IV collagen.

Transcription and Translation (See Figure 1). Heterogeneity in collagen gene products was identified 10 years ago and this heterogeneity is now viewed as a potential site of defective cellular control in a number of connective tissue diseases. Seven collagen α chains with distinct characteristics have been identified from five genetic forms of collagen. The most common are α1(I) and α2, which form the most abundant triple helical collagen as $[\alpha 1(I)]_2\alpha 2$ or type I. Cartilage collagen, type II, contains three identical chains of collagen that are assembled as $[\alpha 1(II)]_3$. Type III collagen was first reported in human fetal skin as $[\alpha 1(III)]_3$ and has since been reported in human aorta and other connective tissues. The composition of basement membrane collagen (type IV) remains in doubt at this time. Additional collagen gene products, identified as αA and αB chains, have most recently been reported as minor components of connective tissues from a variety of tissue sources and are now identified as type V collagen. Since the physical/chemical characteristics and the function of structural collagen types differ, a shift in the ratio of collagen types synthesized by a fibrotic organ may represent a biochemical lesion closely related to the basic disease mechanism.

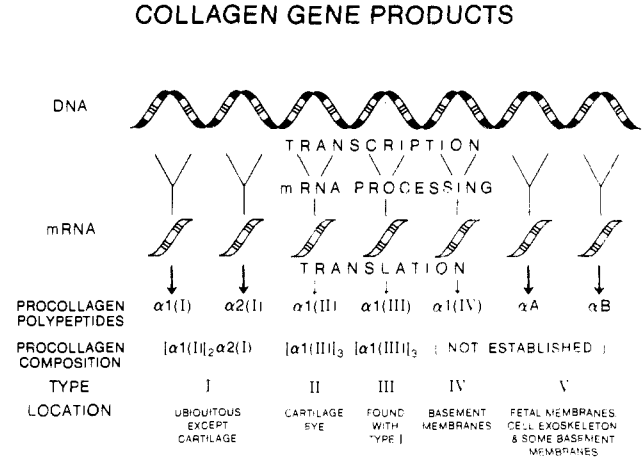


Figure 1.

The existence of a specific and separate mRNA for each procollagen α chain has been confirmed in a number of laboratories by the isolation and translation of specific collagen messages. Several DNA fragments corresponding to sections of the message units have been cloned. These studies have also confirmed that the monomeric precursors of triple helical collagen are synthesized with globular extensions at both the carboxy and amino terminus.

The polypeptide units of collagen are synthesized using conventional translation mechanisms from their respective mRNAs. The procollagen α chains are synthesized on membrane-bound ribosomes, inserted through the microsomal membrane, and processed in the cisternal space of the rough endoplasmic reticulum.

Intracellular Processing (Figure 2). Prolyl and Lysyl Hydroxylase. As nascent chain procollagen enters the cisternae of the endoplasmic reticulum, it is processed by hydroxylating enzymes resulting in the conversion of prolyl residues to 4-hydroxyproline, with some converted to 3-hydroxyproline in the case of type IV and type V collagen. A third hydroxylase results in the conversion of lysyl residues to hydroxylysine. As these substrates are hydroxylated, α-ketoglutarate (a cosubstrate) is stoichiometrically decarboxylated to succinate and CO_2. In addition to this cosubstrate, these enzymes also require molecular oxygen, ferrous ion, and ascorbate.

Galactosyltransferase and Glucosyltransferase. These enzymes transer carbohydrate moieties using the appropriate uridine diphosphate glycoside as a cosubstrate.

(6) Fuller, G. C. In "Connective Tissue of the Normal and Fibrotic Human Liver"; Gerlach, U.; Pott, G.; Rauterberg, J., Eds.; G. Thieme Verlag: Stuttgart and New York, 1981, in press.
(7) Bornstein, P.; Sage, H. *Annu. Rev. Biochem.* **1980**, *49*, 957–1003.
(8) Eyre, D. *Science* **1980**, *207*, 1315–1322.
(9) Bienkowski, R. S.; Cowan, M. J.; McDonald, J. A.; Crystal, R. G. *J. Biol. Chem.* **1978**, *253*, 4356–4263.

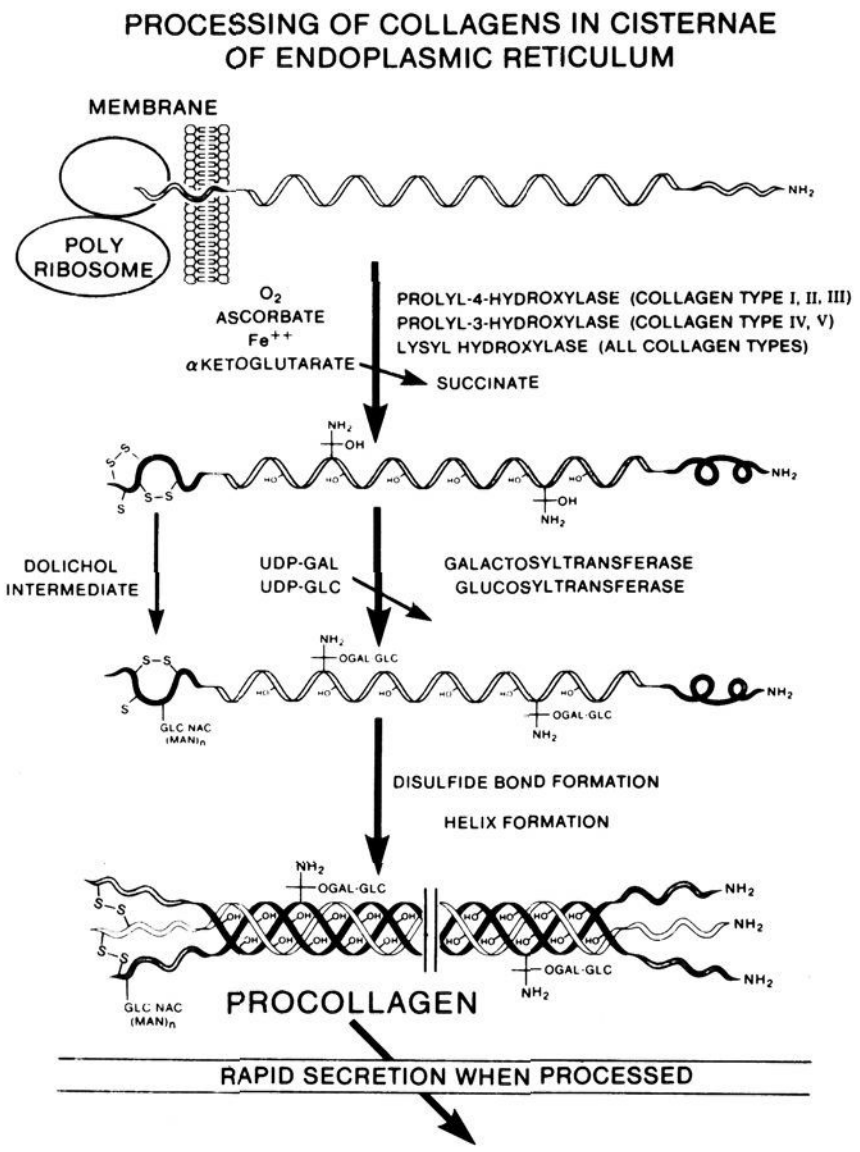


Figure 2.

First, a galactose is added to the hydroxyl group of hydroxylysine followed by the addition of a glucose to the galactose. These glycosylation reactions occur in the cisternae of the rough endoplasmic reticulum and supply the only known carbohydrate moieties in the triple helical region of collagen. Both enzymes require a bivalent cation and a nonhelical substrate. Glycosylation ceases upon triple helix formation. The carbohydrate moieties (mannose) of the nonhelical propeptide regions are added through other enzymatic steps that are thought to require dolichol intermediates. The functional requirement for the glycosylation of collagen remains unknown. However, tissue levels of the transferases, like the other intracellular processing enzymes, increase during periods of enhanced collagen formation and fibrogenesis.

Disulfide Bond Synthesis, Helix Formation, and Secretion. The formation of intrachain and interchain disulfide bonds in the procollagen molecule occurs after translation is completed and probably after the respective procollagen α chains are released from polyribosomes. The interchain bonds formed are located in the carboxypropeptide region of procollagen. The disulfide bonds are subsequently removed with the propeptide, except for collagen type III where the helical portion of this collagen terminates in an area containing disulfide bonds after the propeptide is removed. In addition to hydroxylation of proline residues, the formation of interchain disulfide bonds is essential for helix formation at physiological temperature. Helix formation occurs in the rough endoplasmic reticulum or the Golgi complex where the helical procollagen is packaged into a Golgi vacuole prior to exocytosis.

These cellular assembly and secretion steps are important with regard to the mechanisms regulating extracellular collagen accumulation. The activity of all cellular processing enzymes increase during periods of fibrogenesis.

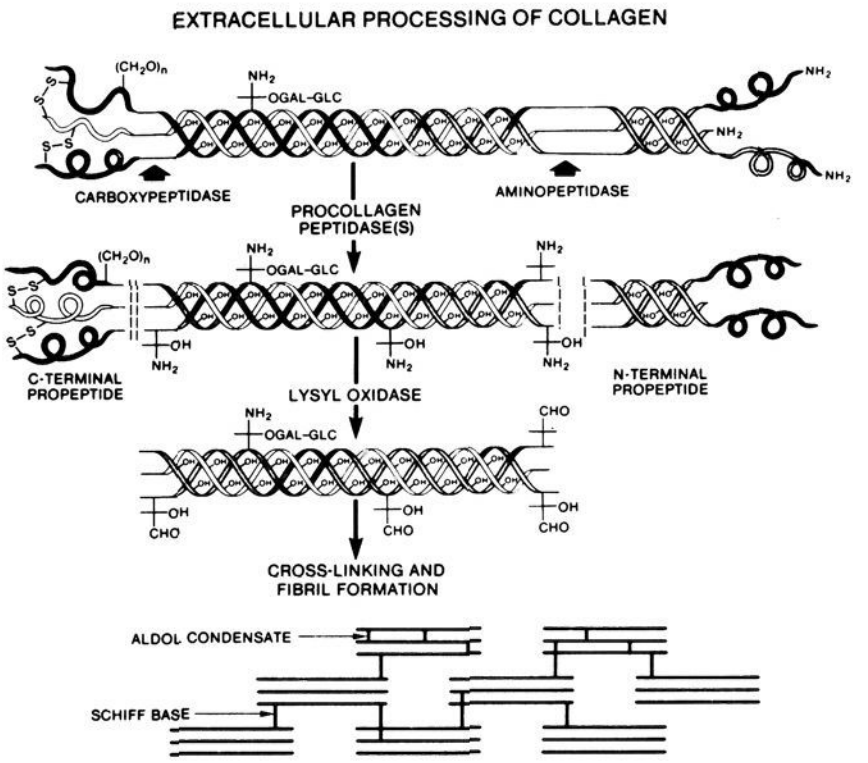


Figure 3.

Recent evidence indicates that 20 to 30% of the hydroxyproline and hydroxylysine synthesized in cultured fetal lung cells is degraded intercellularly to dialyzable peptides and amino acids before it is secreted.[9] This observation may explain the presence of the urinary hydroxyproline found during periods of elevated collagen synthesis and, more important, implicates the rate of cellular processing as a mechanism for regulating the amount of structural collagen that eventually finds its way into the extracellular fibrils. Since the collagen made by cells with impaired processing systems cannot mature into stable fibrils, these cellular enzyme systems become logical targets for drug development programs.

Extracellular Processing (Figure 3). Procollagen Peptidases. The removal of the two terminal nonhelical portions of collagen requires two proteases—one for the amino terminus and one for the carboxy terminus. The first enzyme removes a specific peptide from the amino terminus of each of the three chains in the triple helix. This process also includes cleavage of peptides from the carboxy terminus, but because of the interchain disulfide bonds these three peptides remain as a single unit after removal. While most procollagen is converted to collagen by these extracellular enzymes, a procollagen-like molecule may be one of the structural macromolecules in the basement membrane and some procollagens may be retained on the cell surface to interact with the structural components of the extracellular matrix or to impart specialized surface characteristics to the cells such as nonthrombogenicity.[10] The discovery and characterization of several inborn errors of metabolism where these proteases are deficient have established that procollagen must be processed by these proteases to helical collagen molecules, 3000 Å in length, for the formation of fibrils with structural integrity. However, at present there are no data implicating processing errors by these proteases as a mechanism leading to fibrosis.

Lysyl Oxidase and Cross-link Formation. After the assembly of helical collagen into organized fibrils, covalent bonds are formed within and between helixes which impart the rigidity and stability of interstitial collagen. These covalent bonds occur as a result of the oxidative deamination of either lysyl or hydroxylysyl residues to the cor-

(10) Madri, J. A.; Dreyer, B.; Pitlick, F.; Furthmayr, H. *Lab. Invest.* **1980**, *43*, 303–315.

responding reactive aldehydes—allysine or hydroxyallysine. The aldehydes on adjacent collagen molecules can react either with themselves (in an aldol condensation reaction) or with the terminal group of lysine or hydroxylysine (in an aldemine formation). In the case of the aldol condensation, a reactive aldehyde group remains and this can react with residues in other chains. This latter cross-linking reaction results in a three-dimensional bonding among collagen molecules. The collagen fibrils become at least tenfold more resistant to degradation by mammalian collagenase as a result of these cross-linking reactions.[11]

Several laboratories have purified active forms of the enzyme, lysyl oxidase, responsible for the oxidative deamination of peptidyl lysine in this extracellular processing step. Copper and O_2 are required cofactors, and some of these preparations are also dependent upon pyridoxal phosphate for activity.

Mammalian Collagenase and Collagen Turnover. The turnover and resorption of collagenous tissue occurs normally during growth and development, in wound healing, and under special circumstances such as uterine involution after parturition. Even in the adult, where the rate of metabolic turnover is low, some newly synthesized collagen is demonstrable in tissue such as skin. Thus, the normal synthesis of collagen must be balanced by an equivalent rate of catabolism in order to avoid accumulation and fibrosis. Under physiological conditions of temperature and pH, completely processed collagen is only slightly susceptible to most vertebrate proteolytic enzymes. The turnover of collagen occurs through the activity of mammalian collagenase, which cleaves native collagen at a single site in the helical region of each collagen chain.

Recent reviews have summarized the progress in the mammalian collagenase area and discussed the problems inherent in measuring this enzyme in tissue.[5,12] The principal problems in interpreting mammalian collagenase data are caused by the presence of potent inhibitors of the enzyme in serum and tissue, the role of proposed proenzyme forms, and the binding of enzyme to endogenous substrate, all of which contribute to a large pool of latent enzyme. The cleavage sites in the collagen substrates are characterized but the various collagen types are reported to have variable resistance to collagenase cleavage. In addition to the extracellular modulation of collagenase activity, intracellular regulation of the synthesis and secretion of the proenzyme is also likely.

Normal growth and remodeling depend upon collagenases that can degrade collagen in fibril form or upon other proteases to depolymerize collagen fibers in the collagenolytic process. Both tissue-extracted insoluble polymeric collagen and collagen fibers reconstituted in vitro can be degraded by highly purified preparations of mammalian synovial collagenase and by tumor cell collagenase, although the polymeric fibrils are degraded much more slowly. As pointed out above, highly cross-linked collagens are more resistant to digestion. The mechanistic role of collagenase in the fibrotic process is, thus, difficult to assess because of the variety of enzyme forms, the presence of inhibitors, and the variable resistance of the different forms of collagen to collagenase degradation. The present understanding of these interactions show promise of becoming even further complicated in view of recent suggestions that some forms of collagenases have specificity directed toward single collagen types and that some nonspecific neutral proteases degrade collagen. Nevertheless, changes in collagenase activity in fibrotic organs have been reported and, although their role in fibrogenesis is unresolved, the requirement for collagenase participation in the reversal of fibrosis is unmistakable.

Possible Mechanisms of Fibrosis. It is evident from the preceding discussion that the mechanisms accounting for a fibrotic lesion may reside at one or more of a wide variety of possible regulatory sites normally responsible for the control of collagen synthesis, deposition or matrix formation, and/or the degradation steps responsible for matrix turnover. The earliest prefibrotic change which leads to a fibrotic lesion is likely to be a shift in the cell population of prefibrotic organs toward cells that are fibroblastic in nature, either through recruitment and induced mitogenesis of cells committed to fibroblastic activity or transformation of the normal population of cells to a population with enriched fibroblastic activity. Changes in collagen mRNA levels and the efficiency of its translation are also mechanisms capable of increasing collagen production as well as producing inappropriate ratios of specific gene products. An increase in the cellular processing of collagen remains a viable means of increasing the amount of collagen secreted and, thus, has been the focus of much attention. Increased cross-linking is related to the rate of maturation and increased resistance of fibrils to proteolytic extracellular processing; hence, this might also serve as a mechanism of increased collagen accumulation. Finally, control of degradation by regulating the amount of collagenase or by altering the levels of collagenase inhibitors cannot be excluded as possible biochemical lesions, accounting for fibrosis. A unifying hypothesis describing the mechanism for pathologic collagen accumulation in the variety of tissues that acquire fibrotic lesions remains elusive. There is, however, ample evidence that fibrotic collagen accumulation results from the overproduction of collagen in animal models and in man. Therefore, an agent which can suppress this overproduction of collagen could be expected to intervene in the progression of a fibrotic process and thereby prevent end-stage organ failure.

Sites and Therapeutic Implications of Potential Intervention

As pointed out above, alteration of the collagenous components of the matrix by altering gene expression to result in either an abnormal mixture or amount of a specific collagen type in the matrix is an attractive possibility as a mechanism that could account for fibrosis. However, with the exception of platelet aggregation, where types I–III but not type IV cause aggregation, the structural requirement for each collagen type has not been identified. A number of reports of changes in the synthesis and accumulation of collagen gene products during fibrogenesis have been published (see reviews, ref 3 and 4). Mature scar tissues of skin, tendon, myocardium, and of vascular tissue are now known to contain increased amounts of both types I and III collagen. Earlier reports had suggested that type III collagen may be preferentially increased in liver and lung fibrosis. This suggestion was supported by the observations that type III collagen is produced early in wound healing by primitive mesenchymal-derived cells, with type I synthesis increasing only after the appearance of mature fibroblasts. Because of the absence of definitive data on the role of collagen types I and III in the formation of scar tissue, it is not possible at this time to relate a shift in the collagen type I to III ratios to a defined mechanism that explains why an organ will regenerate after some lesions but develop end-stage fibrosis in response to other forms of cellular injury. However, the available data does

(11) Vater, C. A.; Harris, E. D.; Siegel, R. D. *Biochem. J.* **1979**, *181*, 639–645.
(12) Perez-Tamayo, R. *Am. J. Pathol.* **1978**, *92*, 508–566.

indicate that antifibrotic therapy should focus on the inhibition of both collagen type I and III synthesis and accumulation.

The regulatory site in the collagen biosynthetic pathway remains unidentified at this time. However, the numerous attempts that have been made to identify the rate-limiting step in the synthetic pathway provide conceptual direction for antifibrotic drug development. Mammalian tissue culture systems have been used by a number of investigators to study the influence of a variety of endogenous factors on collagen synthesis. The methodology available has permitted studies that assess changes in rates of synthesis and intracellular processing of collagen gene products with a high degree of precision. Using these systems, a variety of small molecular weight endogenous compounds and a few larger peptide fractions have been identified as potential components of the tissue response leading to fibrosis. These endogenous factors are presumed to act at the translation level because they increase the incorporation of labeled amino acids into collagenous proteins. However, it is not clear if this is a reflection of altered levels of mRNA, altered species of mRNA, or a change in the efficiency of the translation process. The expanding availability of cDNAs which may be used as probes to hybridize specific collagen mRNAs should provide a more definitive answer to this question. Antifibrotic drug development directed toward preventing the effect of these stimulators at the cellular level remains a possibility but one that is premature for development until questions pertaining to their mechanism source, tissue specificity, and role in fibrogenesis are established.

A role for specific propeptide fragments derived from the NH_2-terminal extension peptides of collagen as feedback regulators of collagen synthesis has been proposed and tested in tissue culture.[13] In cultured cells, propeptides of types I and III bovine collagen inhibit the synthesis of type I procollagen by calf or human fibroblasts but do not inhibit the synthesis by type II collagen by chondrocytes. The specificity of these was examined further in cell-free protein synthesis systems using rat mRNA in a reticulocyte system.[14] In this system, the propeptide fragments inhibited the translation of the collagen mRNA but did not inhibit the translation of the message for noncollagen proteins, confirming that the selective inhibition of type I or III collagen required for antifibrotic activity is possible. Radioimmunoassay procedures have been developed to detect and quantitate the levels of these collagen propeptide fragments in serum with the expectation that the titers of these would reflect the amount of new collagen being synthesized. At least one of these, the assay for the amino-terminal peptide from procollagen type III, appears to be of value in detecting fibrogenesis in patients with liver disease.[15] It is apparent that these procollagen peptides will continue to be of interest as research tools, as diagnostic probes, and as models for drug development.

When considering putative mechanisms for influencing the regulation of collagen synthesis at the level of translation, it should be pointed out that the rapid synthesis of collagen imposes an enormous cellular requirement for glycine and for the imino acid proline, which together comprise two-thirds of helical collagen. This fact and the observation that the proline pool size increases in cirrhotic liver and in serum of cirrhosis patients suggest that the rate of collagen synthesis may, in fact, be limited by the availability of proline.[16] This hypothesis, although essentially untested, implies that proline pools are expanded in response to increased demand. However, the cell can make both of these components and, hence, attempts to limit the availability of proline or glycine would require blocking steps in the intermediatory metabolism of the cell. An alternative means of disrupting collagen synthesis at the translation level was suggested by reports from two laboratories that one codon-specific isoaccepting glycyl-tRNA species is used preferentially for the assembly of collagen peptides.[17,18] Blocking the specific codon for such an isoaccepting tRNA would result in the loss of the ability of the cell to translate the mRNA for collagen and, hence, reduce collagen synthesis. The degree of specificity resulting from this approach would depend on the restricted use of this codon-specific tRNA in the synthesis of noncollagenous protein.

As previously noted, the hydroxylation of prolyl and lysyl residues is the first of several processing steps required in the synthesis and secretion of structural collagen. Prolyl 4-hydroxylase has been studied more extensively than the other two hydroxylases and has been the subject of a comprehensive review.[19] Briefly, the active form of this enzyme is a membrane-bound tetramer (240 000 daltons) consisting of two species of inactive monomer which differ in molecular weight. The active enzyme hydroxylates prolyl residues in the -x-Pro-Gly-triplet, but not all available prolyl residues will be hydroxylated. The lack of complete hydroxylation in secreted collagen suggests the presence of a cellular mechanism for controlling the extent of hydroxylation. This may be the rate of helix formation, since the helical conformation of the substrate inhibits hydroxylation. It is also possible that secondary binding sites for the peptidyl substrate exist on the enzyme which function to orient the substrate with the primary binding site and catalytic site. The presence of secondary binding sites would also explain the increase in affinity for substrate which occurs with increased substrate molecular weight.

Several mechanisms have been proposed to account for the coupled decarboxylation of α-ketoglutarate and hydroxylation of prolyl residues.[20] The enzyme is a mixed function oxidase ($^{18}O_2$ is incorporated into hydroxyproline and succinate) which requires Fe^{2+} and ascorbate for activity but does not use ascorbate stoichiometrically. Purified enzyme will decarboxylate α-ketoglutarate at reduced velocity in the absence of peptidyl proline but will not hydroxylate prolyl residues in the absence of α-ketoglutarate. Hydroxylation mechanisms based on the formation of peroxysuccinic acid or hydroperoxide intermediates have been proposed but have not been proven. Siegel[20] has recently proposed a tandom mechanism in which the decarboxylation of α-ketoglutarate is coupled to the production of an activated oxoiron species which, in turn, is coupled to the hydroxylation of prolyl residues. In this proposed mechanism, the iron is coordinated with

(13) Wiestner, M.; Krieg, T.; Horlein, D.; Glanville, R. W.; Fietzek, P.; Muller, P. K. *J. Biol. Chem.* **1979**, *254*, 7016–7023.
(14) Paglia, L.; Wilcsek, J.; de Leon, L. D.; Martin, G. R.; Horlein, D.; Muller, P. *Biochemistry* **1979**, *18*, 5030–5034.
(15) Rohde, H.; Vargas, L.; Hahn, E.; Kalbeieisch, H.; Bruguera, M.; Timpl, R. *Eur. J. Clin. Invest.* **1979**, *9*, 451–459.
(16) Kershenobich, D.; Fierro, F. J.; Rojkind, M. *J. Clin. Invest.* **1970**, *49*, 2246–2249.
(17) Carpousis, A.; Christner, P.; Rosenbloom, J. *J. Biol. Chem.* **1977**, *252*, 8023–8026.
(18) Drabkin, H. J.; Lukins, L. N. *J. Biol. Chem.* **1978**, *253*, 6233–6241.
(19) Cardinale, G. J.; Udenfriend, S. *Adv. Enzymol.* **1974**, *41*, 245–300.
(20) Siegel, B. *Bioorg. Chem.* **1979**, *8*, 219–226.

cysteine residues at the enzyme active site, and these cysteine residues are maintained in a reduced state by the presence of ascorbate. The enzyme will not hydroxylate free proline and requires at least a tripeptide prolyl substrate but binds to high-molecular-weight native substrates with high affinities. Thus, the accumulated information about the enzyme binding sites and the information gained from mechanism studies could provide a basis for initiating the directed synthesis of specific prolyl 4-hydroxylase inhibitors.

Prolyl 4-hydroxylase is one of the primary targets for antifibrotic development because of the critical hydroxyproline requirement for the active secretion of collagen and for the thermal stability of collagen (unhydroxylated collagen cannot assume a helix configuration at 37 °C). Further, inhibition of the 4-hydroxylase would impart a degree of specificity for collagen types I–III and partially spare the basement membrane (type IV) pathway which requires prolyl 3-hydroxylation in addition to prolyl 4-hydroxylation. Finally, the historical or classic accounts of scurvy among sailors included the observation that old wounds or scar tissue became disrupted months after apparent wound closure. Since collagen accumulation is known to be depressed in scorbutic animals, it would follow that the turnover of scar-tissue collagen exceeded that of new synthesis and resulted in the eventual opening of old wounds in the scorbutic sailors. Thus, titrating the dose of a prolyl 4-hydroxylase inhibitor could achieve a therapeutically acceptable level of activity sufficient to suppress the formation of new scar tissue in the fibrosing lung or liver.

The enzyme responsible for prolyl 3-hydroxylation (needed for basement membrane or collagen type IV) has not been characterized to the same extent as prolyl 4-hydroxylase. The affinity constants of the partially purified prolyl 3-hydroxylase for substrates and cofactors (O_2, α-ketoglutarate, Fe^{2+}, and ascorbate) are similar to the 4-hydroxylase.[21] The 3-hydroxylase is a smaller protein (160 000 daltons) and requires proline in the sequence Gly-Pro-4Hyp-Gly for recognition and hydroxylation to occur in the trans-3 position. The development of inhibitors directed against the prolyl 4-hydroxylase would probably lead to the rapid development of prolyl 3-hydroxylase inhibitors because of the similar characteristics of these two enzymes. These inhibitors may be useful in preventing the basement membrane thickening in diabetics, but their application would need to proceed with caution because of the vital nature of these collagens in basement membrane structures throughout the body (blood vessel wall, glomerulus, and lens capsule).

The active purified form of lysyl hydroxylase appears to be a dimeric enzyme composed of two dissimilar subunits.[22] Lysyl hydroxylase requires the same cofactors and cosubstrates for maximal activity as prolyl hydroxylase except for the requirement for peptidyl lysine as Gly-Lys. Hydroxylation of specific lysyl residues is necessary to provide sites for the glycosylation of collagen, and hydroxylysine is a participant in at least one of the extracellular cross-linking reactions. However, the synthesis of cartilage collagen (type II), being enriched in glycosylated hydroxylysine, would be influenced preferentially by inhibitors of lysyl hydroxylase. Therefore, this hydroxylase does not appear to be a highly desirable target for inhibition if the clinical end point is focused on fibrogenesis in the generation of scar tissue.

An endogenous inhibitor of prolyl 4-hydroxylase has been partially characterized.[23] Unfortunately, this inhibitor competes at the α-ketoglutarate binding site. It is active against at least four of the α-ketoglutarate-dependent hydroxylases and is therefore not appropriate as a model for antifibrotic drug development.

Collagen galactosyltransferase and glucosyltransferase are enzymes which transfer the carbohydrate moieties, using the appropriate uridine diphosphate glycoside as the cosubstrate for the addition, first, of a galactose to the hydroxyl group of hydroxylysine in collagen and, next, of a glucose to the galactose. These glycosylation reactions occur in the cisternae of the rough endoplasmic reticulum, require a bivalent cation, and glycosylate only nonhelical substrate. Although genetic deficiencies of these enzymes may account for some degenerative connective tissue disorders,[24] these enzymes cannot be considered primary objectives of an antifibrotic drug development program for the same reasons given for lysyl hydroxylase. It is interesting that considerable quantities of these enzymes are found in serum with 10% of the serum activity bound to platelet membranes. This observation generated considerable interest in the role of this enzyme in collagen–platelet interactions resulting in adhesion or aggregation. The recent observation that platelet-bound enzyme cannot bind the undenatured triple helical collagen which is required for platelet aggregation argues convincingly against such a role for this enzyme.[25]

The formation of intrachain and interchain disulfide bonds in the procollagen molecule occurs after translation is completed and probably after the respective procollagen α chains are relased from polyribosomes. The interchain bonds formed are located in the carboxypropeptide region. The formation of interchain disulfide bonds is essential for helix formation, but it is not clear whether disulfide bond formation in collagen is spontaneous or enzyme directed. Recently, a protein disulfide-isomerase was described, the activity of which is correlated with the biosynthesis of collagen.[26] The enzyme described, however, appears to have broad specificity for protein disulfide bonds and is, therefore, not likely to be a productive target for the development of an antifibrotic agent with specificity for the collagen pathway.

Collagen helix formation occurs in the rough endoplasmic reticulum or the Golgi complex where the helical procollagen is packaged into a Golgi vacuole prior to exocytosis. The secretion of collagen could be blocked as a means of reducing extracellular collagen, but again this approach would suffer from a lack of specificity since the secretion of all exported proteins would be prevented.

The extracellular processing of the helical procollagen includes the removal of the two terminal nonhelical extension propeptides by two proteases—one for the amino terminus and one for the carboxy terminus. These enzymes were discovered with the characterization of inhertied disorders in which they are absent or deficient. The amino-terminal propeptidase has been extensively characterized,[27] and peptide inhibitors homologous to the cleavage site in procollagen have been described.[28] How-

(21) Trygguason, K.; Majamaa, K.; Risteli, J.; Kivirikko, K. *Biochem. J.* **1979**, *183*, 303–307.
(22) Miller, R. L.; Varner, H. H. *Biochemistry* **1979**, *18*, 5928–5932.
(23) Chichester, C. O.; Fuller, G. C. *Res. Commun. Chem. Pathol. Pharmacol.* **1980**, *29*, 329–338.
(24) Savolainen, E. R.; Kero, M.; Pihlajaniemi, T.; Kivirikko, K. I. *N. Engl. J. Med.* **1981**, *304*, 197–204.
(25) Menashi, S.; Grant, M. E. *Biochem. J.* **1979**, *178*, 777–784.
(26) Brockway, B. E.; Forster, S. J.; Freedman, R. B. *Biochem. J.* **1980**, *191*, 873–876.
(27) Nusgens, B. V.; Goebels, Y.; Shinkai, H.; La Piere, C. M. *Biochem. J.* **1980**, *191*, 699–706.

ever, helical collagen containing the amino-terminal propeptides appears to form functional fibrils, whereas helical collagen with the carboxy-terminal propeptides cannot be packed into fibrils presumably because of the size of the propeptides. This means that the carboxy-terminal procollagen peptidase would be the preferred peptidase to inhibit as a means of preventing fibrogenesis. Unfortunately, this enzyme is not sufficiently characterized at the present time to allow for expedient inhibitor development.

After the assembly of helical collagen into organized fibrils, covalent bonds are formed within and between helixes which impart the rigidity and stability of interstitial collagen. Those covalent bonds can be formed only after the oxidation deamination, by lysyl oxidase, of either lysyl or hydroxylysyl residues to the corresponding reactive aldehyde—allysine or hydroxyallysine. These aldehydes then participate in the cross-linking reactions.

Several laboratories have purified active forms of lysyl oxidase.[29] The various preparations of lysyl oxidase have been purified in a wide range of molecular weights with multiple peaks of activity found from most tissue sources. However, they are all active in the oxidation deamination of ϵ-amino groups of specific lysyl residues in both collagen and elastin, and all are inhibited by 4–10 μM β-aminopropionitrile. There also is evidence that some of these preparations are dependent upon pyridoxal phosphate for activity.

Increased lysyl oxidase activity has been reported in various forms of scar tissue and these increases have been observed to persist in scars for up to 5 years. Animals with experimentally induced liver fibrosis have increased lysyl oxidase activity of up to 30-fold in liver tissue and 15-fold in serum. Based on these observations, it has been suggested that lysyl oxidase may be a marker for the rate of extracellular deposition (see reivew, ref 29). It is now clear that collagen fibrils that are cross-linked through these reactions become at least ten times more resistant to degradation by mammalian collagenase as a result, suggesting that levels of lysyl oxidase may be important not only in determining or explaining differences in the rates of extracellular maturation of various collagen types but also in influencing the degree of reversibility of the fibrotic lesion. It would also follow that inhibition of lysyl oxidase would provide control of aggressive collagen deposition during fibrosis and, indeed, this has been demonstrated (see review, ref 6). Unfortunately, lysyl oxidase has broad substrate specificity for lysyl residues in both collagen and elastin[30] and will even deaminate nonpeptidyl amines.[31] Therefore, it is unlikely that the lathyrogenic effects associated with the inhibition of elastogenesis could be avoided in an inhibitor of lysyl oxidase developed as an antifibrotic agent.

Projected Role of Antifibrotic Therapy

The clinical experience gained through the use of proline analogues and inhibitors of collagen cross-linking as a means of preventing the aggressive deposition of collagen in rapidly progressing fibrosis suggest several criteria for the development of antifibrotic agents.

(1) Specificity for the collagen pathway. This appears necessary in view of the adverse effects demonstrated by inhibitors of cross-linking on elastogenesis and the adverse effect of the proline analogues (in singlet form) on noncollagen protein synthesis.

(2) Specificity within the collagen pathway for collagen types I and III. Because of the critical requirement for basement membrane collagens as a functional component of several vital organ systems (blood vessels, kidney, and eye), the synthesis of collagen type IV should not be influenced by antifibrotic agents. Similarly, the effect of antifibrotic therapy on collagen type II (cartilage) should be minimal, since this gene product is clearly not a participant in the fibrotic response of parenchymatous organs.

(3) A dose–response curve which allows the clinical titration of dose against the appropriate parameter of fibrogenesis. It would be irresponsible to deal with fibrogenesis through total supression of collagen formation, since some new synthesis is constantly required to maintain structural integrity, especially in pressure-bearing or load-bearing organ systems. The clinical objective of antifibrotic therapy is the reduction, to normal levels, of the large increases in collagen synthesis and processing which have been shown to occur in the prefibrotic and early fibrotic stages of scar tissue formation. Thus, the successful management of fibrosis will also depend on the development of chemical markers for pathway activity and their clinical application. There has been considerable expansion of research recently in this specific diagnostic area because of the need to identify which fibrosis patients are progressing to end-stage fibrosis. Several assay systems capable of providing these clinical assessment data are presently being evaluated. The selection of the appropriate antifibrotic monitoring parameter will depend on the site of activity of the drug in the collagen pathway.

These criteria would be satisfied by agents with activity directed at any one of several sites in the collagen pathway. These include the following.

(1) Agents developed to block the stimulatory effect of endogenous mediators of the pathway which are presumed to be involved in the etiology of postinflammatory fibrosis. These mediators are not sufficiently characterized at this time to allow for rational drug development and, further, their origin and characteristics may vary from organ to organ. Drugs developed to offset the stimulatory effects of these mediators would be highly specific for a particular fibrogenic lesion; therefore, this approach should become applicable for the development of second-generation antifibrotic agents in the future.

(2) Exogenous compounds which mimic the feedback inhibitory effect of the procollagen extension peptides on collagen synthesis. The endogenous peptides which would serve as models for the development of these compounds appear to be specific for the inhibition of collagen types I and III, but the mechanism by which these inhibit the translation of procollagen mRNA is not known at the present time. Immunoassay procedures for the quantitation of collagen propeptides in serum or urine have been developed and could be used to assess the effect of these drugs on collagen biosynthesis. The principal challenge with this type of agent will be the development of a biologically stable compound which has the desired dose–response characteristics and a reasonable duration of action.

(3) Inhibitors of prolyl 4-hydroxylase would suppress the processing and, therefore, the extracellular accumulation of collagen types I–III. Inhibition of collagen type II synthesis would be an undesirable side effect but probably not a limiting feature because of the relatively slow turnover of cartilage. The clinical effect of these inhibitors could be monitored either by direct enzyme assay (in the

(28) Morikawa, T.; Tuderman, L.; Prockop, D. J. *Biochemistry* **1980**, *19*, 2646–2650.
(29) Siegel, R. C. *Int. Rev. Connect. Tissue Res.* **1979**, *8*, 73–118.
(30) Kagan, H. M.; Tseng, L.; Trackman, P. C.; Okamoto, K.; Rapaka, R. S.; Urry, D. W. *J. Biol. Chem.* **1980**, *255*, 3656–3659.
(31) Trackman, P. C.; Kagan, H. M. *J. Biol. Chem.* **1979**, *254*, 7831–7836.

case of lung washings) or by the presence of urinary hydroxyproline which increases during periods of active collagen synthesis. The immunoassay procedures developed for the hydroxylase will probably not be useful here because they detect both inhibited and uninhibited enzyme. The amount of propeptide fragments formed and detected by the immunoassay for these would probably remain unchanged by a prolyl 4-hydroxylase inhibitor because the de novo pathway would not be suppressed but rather the products of the pathway, by failing to reach maturity, would be degraded immediately. The primary obstacle faced in the development of prolyl hydroxylase inhibitors is the high affinity of the enzyme for its endogenous peptidyl substrate. Successful inhibitors will need to gain access to the intracellular enzyme in concentrations sufficient to prevent enzyme–substrate interactions and, because of the kinetics of the system, will probably require very low inhibition constants to be active in vivo.

(4) Inhibitors of the procollagen peptidases would suppress collagen fibril formation and, therefore, prevent the accumulation of degradation-resistant forms of collagen in scar tissue. The specificity of antifibrotic agents directed toward these enzymes will become more apparent as the specific proteases are characterized. An inhibitor of a procollagen amino-terminal protease based on the amino acid sequence around the cleavage site in the proα1 chain of type I collagen has been described.[28] However, the procollagen carboxy-terminal peptidase, which would be the primary objective of an antifibrotic development program, has not been characterized. Thus, inhibitors of the procollagen peptidases would have to be considered at this time as possible second-generation antifibrotic agents.

(5) Selective activators of mammalian collagenase may be ultimately useful in resolving preformed scar tissue. The four types of activity described above might serve as the basis for a drug to prevent the progression of a fibrotic lesion. The reversal of an existing fibrosis may, in fact, occur as a consequence of suppressed collagen accumulation since turnover of collagenous protein is continuous. Any reversal that occurs will clearly require the participation of an active collagenase. It would follow that the remodeling process could be accelerated by the selective activation of mammalian collagenases in the fibrosed organ. However, the regulation of mammalian collagenase is not understood sufficiently at present to identify the feasibility of this approach.

Inhibitors of collagen synthesis, including those with defined specificity for a prefibrotic or fibrotic lesion, would produce predictable toxicity if collagen synthesis were inhibited systemically. All known agents which influence connective tissue formation are teratogens. In addition, collagen domains (helical protein structures containing Pro-Hyp-Gly sequences) are found in a number of proteins. The impact of a collagen synthesis inhibitor on the function of these proteins cannot be assessed with accuracy until a useful inhibitor is available for systemic use. The proteins which may be influenced by inhibiting collagen synthesis or, more likely, by processing include the first component of complement (Clq), which contains six triple helical domains per molecule,[32] and acetylcholinesterase, where a collagenous tail has been implicated in the anchorage of this protein to the cell membrane matrix.[33] In view of the life-threatening nature of progressive fibrosis in organs like lung, liver, and kidney, the risks accepted with the use of an effective antifibrotic therapy would be justified if the dose–response curve of the drug provided reason to believe that the impact on these and other collagen-dependent systems could be minimized.

(32) Reid, K. B. M.; Porter, R. R. *Biochem. J.* **1976**, *155*, 19–23.
(33) Rosenberry, T. L.; Richardson, J. M. *Biochemistry* **1977**, *16*, 3550–3558.

Mesoionic Xanthine Analogues: Phosphodiesterase Inhibitory and Hypotensive Activity

Richard A. Glennon,* Michael E. Rogers, J. Doyle Smith, M. K. El-Said,

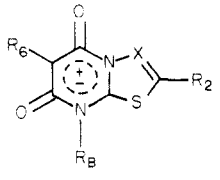
Department of Pharmaceutical Chemistry

and John L. Egle

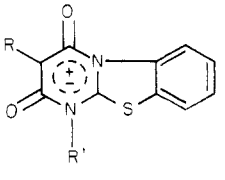
Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298.
Received January 29, 1981

Several mesoionic thiazolo[3,2-*a*]pyrimidines and mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidines were evaluated as inhibitors of cyclic-AMP phosphodiesterase. While small alkyl substituents at the 6 position have no significant effect on activity, phenyl and benzyl substituents enhance activity. Mesoionic structures such as 1 (R_2 = H; R_8 = Et) possess 20 to 40 times the activity of theophylline when the R_6 substituent is phenyl or 4-chlorobenzyl. Methyl and ethyl substitution at the 2 position essentially abolishes activity. Although plagued by solubility problems, several of the mesoionic derivatives were found to display weak hypotensive effects in vivo.

We have previously reported that derivatives of mesoionic 1,3,4-thiadiazolopyrimidines, 1, and mesoionic thiazolopyrimidines, 2, possess theophylline-like activity as inhibitors of adenosine cyclic 3′,5′-monophosphate (cyclic-AMP) phosphodiesterase (PDE).[1] Although the observed activity is rather low, this was the first demonstration that mesoionic xanthine analogues possess such activity. It appears that lengthening and branching of alkyl chains at the 8 position (R_8) may enhance potency



1, X = N
2, X = CR



3

(1) Glennon, R. A.; Rogers, M. E.; Bass, R. G.; Ryan, S. B. *J. Pharm. Sci.* **1978**, *67*, 1762.

0022-2623/81/1824-0658$01.25/0