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CONFERENCE REPORT†

ELASTASE INHIBITORS FOR TREATMENT OF EMPHYSEMA

APPROACHES TO SYNTHESIS AND BIOLOGICAL EVALUATION

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INTRODUCTION

Human neutrophil elastase is a protease capable of hydrolysing most connective tissue components, although its most likely primary physiological substrate is elastin. Since destruction of elastin and the concomitant loss of elastic recoil in the emphysematous lung have been well established, attention has been focused on elastase as the primary destructive agent in the pathogenesis of emphysema. On the basis of experimental evidence, it is postulated that increased proteolysis, especially elastolysis, may occur in the lung parenchyma as a result of an imbalance between elastase and its major inhibitor, alpha-1-protease inhibitor, either due to an increased release of the enzyme in the lung or from an acquired or inherited deficiency of the protease inhibitor.

Cigarette smoke, which has been shown to oxidatively inactivate alpha-1-protease inhibitor *in vitro*, is believed to cause a localized functional deficiency of the protease inhibitor in the lungs of smokers. This breakdown of the antiprotease shield in lungs is thought to be a primary factor in the pathogenesis of centrilobular emphysema associated with cigarette smoking. This acquired functional deficiency of the protease inhibitor is transient and has been shown to reverse on cessation of cigarette smoking.

Some individuals (0.02–0.03% of U.S. population) are genetically deficient in alpha-1-protease inhibitor. This deficiency results from inheritance of a defective structural gene, the Z gene, for the protease inhibitor. Alpha-1-protease inhibitor, synthesized under the direction of the Z gene, is poorly secreted from liver cells where it is produced. The alleles are codominant and hence the heterozygosity (i.e. one defective and one normal allele) leads to partial deficiency (about 30–40% of the normal serum concentration) whereas homozygosity (i.e. both defective alleles) leads

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to severe deficiency (10–15% of the normal serum concentration) of alpha-1-protease inhibitor in the plasma as well as in the lungs. Thus the genetic defect leads to a permanent systemic deficiency for the protease inhibitor, and has been linked with the development of the familial or panacinar variety of emphysema, which constitutes about 3% of all emphysema cases in the U.S.

The protease–antiprotease hypothesis for pathogenesis of emphysema provides promising approaches for limiting the damage to lung tissue, at least in early stages of the disease. One approach is to increase the antiprotease levels in the lung by supplementing with suitable elastase inhibitors and thus restoring the protease–antiprotease balance. To achieve this goal, considerable research effort has been directed towards the study of elastase inhibitors, both natural and synthetic, for possible use in treatment of familial emphysema.

To review the research related to design, synthesis and biological evaluation of elastase inhibitors, its state-of-the-art, problems and prospects, and to develop recommendations for future directions, the Division of Lung Diseases of the National Heart, Lung, and Blood Institute conducted a workshop in Bethesda, Maryland, in June 1985. The participants included both industrial and academic scientists, representing a wide range of backgrounds including enzymology, synthetic medicinal chemistry, protein crystallography and molecular modeling, as well as pulmonary biology, physiology, and medicine. The following is a summary of the proceedings of the workshop.

ACTIVE SITE OF ELASTASE

Human neutrophil elastase belongs to the family of serine proteases, along with more widely studied proteolytic enzymes such as trypsin, chymotrypsin, and porcine pancreatic elastase. Since the amino acid sequence and X-ray crystallographic characteristics of human neutrophil elastase are still unreported, a working model of the catalytic site of the enzyme has been deduced from structural and mechanistic studies that have been carried out on other serine proteases. Thus, the classification of human neutrophil elastase as a serine protease has been extremely valuable in providing a basis for rational design of potential physiologically useful inhibitors.

Detailed kinetic studies with human neutrophil elastase have shown that it exhibits some rather unusual features. The amino acid sequence and the length of the peptide chain of the substrate seem to determine whether the enzyme utilizes the entire catalytic apparatus or simply a portion of it. The sequence and the substrate chain length are also found to affect the rate-determining step in the catalytic mechanism.

Currently, amongst other approaches, two powerful techniques, nuclear magnetic resonance (n.m.r.) and site-specific mutagenesis, are being applied to elucidate the relationship of structure to function in the serine protease family of enzymes. For example, n.m.r. studies of the bacterial alpha-lytic protease, with its active site histidine residue labeled with ^{15}N, have demonstrated that the active site hydrogen bond network is differentially affected by various types of inhibitors and that some types of inhibitors (e.g., boronic acids) will form tight complexes with the histidine residue.

Site-specific mutagenesis has been used to alter specific base(s) in the cDNA for trypsin and hence to alter a specific amino acid residue in both the primary substrate

binding site and the catalytic site of the enzyme. Further kinetic studies of these and related mutant enzymes are needed to evaluate the contributions of specific amino acid residues in the active site to the substrate specificity and catalytic efficiency of serine proteases. The information obtained on the nature of the active site and the catalytic mechanisms involved should be valuable for designing new inhibitors of human neutrophil elastase.

SYNTHETIC INHIBITORS

A wide variety of inhibitors have been developed for serine proteases. These include affinity-label inhibitors (active site directed, irreversible inhibitors), mechanism-based inhibitors (enzyme activated, irreversible inhibitors or suicide inhibitors) and tight-binding, reversible inhibitors. At present only a few of these classes of inhibitors have been developed for human neutrophil elastase. Some investigators use the more available chymotrypsin or porcine pancreatic elastase as model enzymes for the development of inhibitors of the human neutrophil elastase, since the active sites of all these enzymes are thought to have a similar sequence and organization. However, there may be significant differences and studies on the active site of human neutrophil elastase should be specifically conducted to help design specific inhibitors for this enzyme.

Affinity-labels react covalently with an active site residue. Among the structures that were described at this workshop are fluorophosphonates and phosphonamides which are prototype amino acid "nerve gas" analogs, and peptide fluoroketones which are tight-binding and slow alkylating inhibitors. It should be noted, however, that none of these inhibitors has been tested with human neutrophil elastase. One group of affinity-label inhibitors (e.g. carbamyl imidazoles and related heterocyclic structures, and azapeptide derivatives) which has been studied for inhibition of human neutrophil elastase, was found to block the enzyme by carbamylating the active site serine residue of the enzyme. Although many of these synthetic inhibitors are hydrolytically unstable, they provide useful models for synthesis of more stable inhibitors.

In contrast to affinity-labels, mechanism-based inhibitors (all of which are heterocyclic compounds) do not usually contain a reactive functional group until the inhibitors are activated by the target enzyme. After the inhibitor binds to the enzyme, the first step in the inhibition reaction is ring-opening by the active site serine to form an acyl enzyme, followed by the generation of a reactive species such as an acid chloride, alkylating agent or another electrophile capable of reacting with a suitably positioned nucleophile in the active site. The modified inhibitor then has the potential for covalent bonding with a nearby amino acid residue in the enzyme. Alternatively, the enzyme acylation could liberate a charged group capable of disrupting the hydrogen bonding network involved in the proton transfer necessary for deacylation and regeneration of the active enzyme. Many of the currently available inhibitors of this type are capable of inactivating serine proteases for long periods of time.

The mechanism-based inhibitors discussed at the workshop included haloenol lactones, chloropyrones, isocoumarins, and ynenol lactones. Haloenol lactones are structures which acylate a serine protease and release a tethered haloketone functional group. The nature of the substituent(s) on the lactone ring system determines the

activity, specificity, and binding constant of the inhibitor. The factors which determine the kinetics of this class of enzyme-activated, irreversible inhibitors include a binding constant and three rate constants, one each for inactivation, catalytic hydrolysis or spontaneous hydrolysis of the initial acyl enzyme. Chloropyrones and chloroisocoumarins both contain a masked acid chloride functional group. In the case of the chloropyrones, the acid chloride, generated after ring opening, was initially thought to react with an enzyme nucleophile. However, current evidence suggests that the acid chloride hydrolyzes to the free acid which forms a stable salt link with the imidazole ring of the histidine residue that is part of the active site of the enzyme. In contrast, chloroisocoumarins may be acylating the active site histidine, but more conclusive evidence will be required to prove this mechanism. Other isocoumarin derivatives were described which yielded reactive quinoneimine methide intermediates upon enzyme acylation. This may then covalently react with the histidine residue. Ynenol lactones are another class of compounds which liberate a reactive allenenic ketone upon reaction with elastase.

Whether one uses affinity-labels or mechanism-based inhibitors the goal is to achieve irreversible or at least long term inhibition of the enzyme. This is precisely the mechanism for inhibition of elastase by alpha-1-protease inhibitor.

Tight binding, reversible inhibitors of human neutrophil elastase are considered by some investigators to have greater therapeutic potential since they are less likely to react with other proteins or nucleophiles *in vivo*. Examples of this class of inhibitor include peptide ketoesters and peptide boronic acids. The ketoesters have peptide bond modifications which inhibit elastase by reacting with the active site serine to form tetrahedral intermediates, similar to those formed upon inhibition of serine proteases by peptide aldehydes. This mimics a transition state or an intermediate that develops during the reaction of the enzyme with substrate and thus forms a stable enzyme-inhibitor complex. Peptidyl boronic acids are also transition-state inhibitors for serine proteases and certain tetrapeptide analogs are very potent inhibitors of human neutrophil elastase. Kinetically, they show an unusual two state, slow binding behavior. The best of the boronic acid inhibitors appears to be relatively non-toxic and to have sufficient *in vivo* stability. These inhibitors have been shown to protect lungs of experimental animals from acute injury induced by intratracheal instillation of porcine pancreatic elastase.

Benzoic acid derivatives containing long hydrocarbon chains are moderate non-competitive inhibitors of human neutrophil elastase. These compounds are patterned after oleic acid, which is also an inhibitor of neutrophil elastase. In both dogs and monkeys, benzoic acid derivatives show good bioavailability and are currently being assessed for safety.

ENZYME: INHIBITOR INTERACTION – MOLECULAR MODELING

A wealth of structural detail has become available from X-ray crystallographic and molecular modeling studies of porcine pancreatic elastase and other serine proteases. This information should aid in developing inhibitors with increased specificity by predicting the best substitution pattern required on the various heterocyclic irreversible inhibitors. However, the impact that these techniques will have on the design of inhibitors of human neutrophil elastase will be muted until a high resolution structure of the enzyme becomes available.

Several investigators are now conducting X-ray crystallographic studies of porcine pancreatic elastase. The mechanism-based inhibitor 3-methoxy-4-chloro-7-aminoisocoumarin has been shown to acylate the active site serine of the enzyme. Quite surprisingly, however, it is also found to react with the acetate anion present in the buffer, with displacement of chloride at C-4, probably via a quinoneimine methide. Crystallographic studies at neutral pH will be required to demonstrate whether the inhibitor also reacts with other active site residues. A crystallographic study of porcine pancreatic elastase to which a peptide chloromethylketone has been bound at the active site has demonstrated that the peptide binds to the enzyme in the same manner as has been observed with chymotrypsin and trypsin.

Molecular modeling and energy calculations have been used to study the interaction of 6-chloro-2-pyrones with the active site of chymotrypsin. These techniques can be used to predict inhibitory potency, but not the actual inhibition constants of a series of related compounds. Since the calculations include no water molecules and are based on a truncated enzyme structure, they are only a rough approximation of true inhibitory events. The techniques are likely to improve significantly in the future.

The crystal structures of complexes of trypsin with a variety of protein inhibitors have been determined. Intimate contacts between the two proteins, the enzyme and the inhibitor, and an extensive hydrogen-bonding network serve to stabilize the complex. Many proteins function as inhibitors, without being rapidly hydrolyzed by proteases, possibly because the interaction between the protease and inhibitor constrains the scissile bond such that the amine leaving group cannot be protonated for departure from the tetrahedral intermediate normally formed in the course of acylation.

PROTEIN INHIBITORS

Alpha-1-protease inhibitor, the major plasma inhibitor of human neutrophil elastase, is a 54 kDa glycoprotein. Synthesized in the liver, this protein diffuses into the lungs where it serves as the major inhibitor protecting lungs from proteolytic enzymes, including elastase, released from inflammatory cells. The normal plasma concentration of this inhibitor is 150–250 mg/dl. In persons with genetic deficiency, related to homozygous ZZ genotype (i.e. two defective alleles), the plasma concentrations of alpha-1-protease inhibitor are less than 15% of the normal. Such persons are found to be susceptible to the development of emphysema at an early age, especially if they are cigarette smokers.

Replacement therapy with alpha-1-protease inhibitor purified from human blood has been suggested as an approach to ameliorate the genetic deficiency. Recent studies have demonstrated that intravenous infusion of appropriate amounts of purified alpha-1-protease inhibitor into the genetically deficient individuals once a week results in the establishment of serum and lung levels of the protease inhibitor thought to be protective. However, the clinical efficacy of the replacement therapy, in terms of its ability to reduce the rate of decline of pulmonary function in patients with varying degree of emphysema, remains to be established. Currently, the blood derived alpha-1-protease inhibitor is being evaluated for its safety in humans.

Other protein inhibitors of elastase from nonhuman sources might provide possible alternatives to plasma derived alpha-1-protease inhibitor for replacement therapy. For example, several investigators are evaluating the usefulness of alpha-1-protease

inhibitor produced by recombinant DNA technique in microorganisms. The protease inhibitor thus produced is not glycosylated, but shows antiprotease activity. Its half life in plasma in laboratory animals is reported to be very short. Whether this non-glycosylated form of alpha-1-protease inhibitor has a longer half life in the lungs and would thus be useful as a drug for direct delivery to the lower airways remains to be proven.

Recently, a genetically-engineered mutant of the gene for alpha-1-protease inhibitor, which contains a valine residue at position 358 in the active site in place of methionine, has been cloned and successfully expressed in yeast and bacteria. This mutant alpha-1-protease inhibitor has anti-elastase activity and it also is resistant to inactivation by oxidation. Safety and efficacy studies to establish the usefulness of this product for possible use in the treatment of emphysema have not been reported.

Eglin C, a protease inhibitor from a medicinal leech is another elastase inhibitor being studied. It is a small peptide with a molecular weight of 8.1 kDa. It shows a very high affinity for human neutrophil elastase, with which it forms a tight complex. The gene for eglin C has been synthesized and the peptide has been produced by recombinant DNA technique in microorganisms. Its toxicity and immunogenicity have not been evaluated.

There is evidence from *in vitro* experiments that elastase, once it is bound to its natural substrate elastin, is not accessible to high molecular weight inhibitors. The low molecular weight inhibitors, on the other hand, may not have this limitation. Therefore, results of *in vivo* studies with alpha-1-protease inhibitor or other high molecular weight inhibitors may not be useful predictors of the possible *in vivo* activity of low molecular weight inhibitors and vice versa.

ANIMAL MODELS FOR EVALUATION OF ELASTASE INHIBITORS FOR TREATMENT OF EMPHYSEMA

Various models of pulmonary emphysema have been developed in a number of laboratories. Some of the approaches employed for induction of emphysema in hamsters, dogs, rats, and mice include: enzymatic (proteolytic) induction; oxidant induction; induction using nutritional modifications, including copper deficiency, galactosamine, or lipopolysaccharide administration.

At the present time, it is unclear which animal model is physiologically most relevant to human emphysema. Some of these procedures (e.g. intratracheal instillation of elastase) induce moderate to severe emphysema in a very short time (96 hours to 1 week) while other procedures (e.g., NO_2 exposure or cigarette smoking) result in the development of mild emphysema in 6 weeks to 5 years. Some of the approaches have no effect on the function of alpha-1-protease inhibitor, while others (e.g., chloramine-T administration) dramatically reduce alpha-1-protease inhibitor function. Thus, the balance between elastase and alpha-1-protease inhibitor must be evaluated in each type of model. Histologic, morphometric, as well as physiologic evaluations are necessary to assess the severity and distribution of emphysema. Of course, an ideal animal model of emphysema, to be useful for evaluation of drugs for the treatment of the human disease, should express all the characteristics of the disease, including a progressive development of the emphysematous lesion.

Administration of *E. coli* endotoxin and D-galactosamine to rats results in numerous biochemical and cellular changes within 24 hours after administration. The

endotoxin induces rapid migration of neutrophils into the pulmonary capillaries and D-galactosamine reduces alpha-1-protease inhibitor production by the liver. Ten weeks after repeated D-galactosamine and endotoxin administration there is a measurable reduction of elastin in lung parenchyma and a significant increase in mean linear intercept and pulmonary compliance. This experimental approach could provide a useful animal model to test the effectiveness of elastase inhibitors in the treatment of emphysema.

The hamster model of emphysema, induced with intratracheal instillation of elastase, has been extensively used for the study of the pathogenesis of the disease and for the evaluation of elastase inhibitors. A comparative *in vivo* study of three elastase inhibitors, alpha-1-protease inhibitor, eglin C, and a chloromethyl ketone, in the hamster model was reported. Each inhibitor was administered to anaesthetized hamsters, followed in 1 hour by administration of human neutrophil elastase. Eight weeks later, lung volume measurements and anatomical studies were carried out. Eglin C was found to be slightly more effective than alpha-1-protease inhibitor, while the chloromethyl ketone was much less effective in providing protection against human neutrophil elastase induced lung injury. Eglin C, given in sufficiently large amounts, was found to provide adequate protection against lung injury induced by human neutrophil elastase, even when the latter was administered eight hours after the administration of the inhibitor. No studies were reported in which the inhibitor was effective when administered after the administration of elastase.

One difficulty in evaluating the *in vivo* effectiveness of the elastase inhibitors is that no accurate methods or biochemical markers are available to quantitate the extent of elastin damage in lungs. Measurement of desmosine, a crosslinking amino acid unique to elastin, have not provided quantitative correlation with the extent of emphysematous changes in lungs. Methods which could accurately determine the slow progression of the emphysematous lesion and its arrest by therapeutic intervention are needed for the evaluation of potential efficacy of candidate drugs.

ISSUES RELATED TO *IN VIVO* EVALUATION OF ELASTASE INHIBITORS

The mode of administration of an elastase inhibitor would determine whether experimentally or therapeutically useful levels of the antielastase drug could be achieved in lungs. Factors such as tissue targeting of the drug, its solubility and diffusion into alveolar interstitium, as well as its stability *in vivo* will have to be considered in this regard.

Attachment of an elastase inhibitor to a compound which would concentrate in the lung or would help trap the drug in the lung interstitium should help target the drug to the lung. Some elastase inhibitors may have little utility because of their intrinsic instability in water, or very short physiologic half life due to rapid excretion. Such compounds may nevertheless be useful if they can be delivered directly to the lung. The efficacy of administration of such drugs, in the form of aerosol, to the lower airways and the eventual diffusion of the drug to the interstitium will have to be investigated.

An important consideration in the choice of an elastase inhibitor for therapy relates to its *in vivo* half life. For example, both tight-binding and irreversible inhibitors are thought to have therapeutic potential. The differentiation between tight-binding

inhibitors and irreversible inhibitors becomes less important as the residence time of a reversible inhibitor approaches the turnover time of the enzyme.

It is probably important that antielastases be resistant to oxidants released directly or indirectly in the biological systems under study. For example, oxygen metabolites (H_2O_2), oxygen-derived free radicals (superoxide anion, hydroxyl radical), and spin-altered states of oxygen (singlet oxygen) are liberated by activated human neutrophils concurrently with the release of elastase. It should be noted that oxidative inactivation of alpha-1-protease inhibitor due to cigarette smoking is thought to be caused by the oxidants present in the cigarette smoke.

It is clear that several interesting inhibitors of human neutrophil elastase are currently available and others are being designed from knowledge gained from existing studies. However, before any of these elastase inhibitors could be considered for clinical use, their safety will have to be established both in animals and in humans. Also prior to their evaluation for safety in humans, the elastase inhibitors will have to be evaluated for efficacy to treat emphysema in appropriate animal models. Ultimately, clinical efficacy of safe elastase inhibitors proposed as drugs will need to be assessed in a clinical trial.

RECOMMENDATIONS

(1) X-ray crystallographic and other structural studies of the active site of human neutrophil elastase (including the binding of inhibitors to the enzyme) should be promoted. Structural information of this type will be invaluable for the design of elastase inhibitors.

(2) Small businesses or other suitable organizations should be encouraged to provide reliable, high purity human neutrophil elastase and elastase substrates at reasonable cost to facilitate studies on the enzyme.

(3) Development of better animal models of emphysema should be encouraged.

(4) Studies of biochemical markers for emphysema, which will help in evaluation of the effectiveness of elastase inhibitors *in vivo*, should be promoted.

(5) An inexpensive method for quantitating alpha-1-protease inhibitor levels in newborn infants should be developed. Identification of infants with genetic deficiency for alpha-1-protease inhibitor could help to eliminate or minimize the exposure of such individuals to secondary risk factors such as cigarette smoke.

(6) Excessive proteolysis, especially elastolysis, is associated with a number of diseases such as pulmonary emphysema, acute respiratory distress syndrome, and several skin disorders. A workshop of clinical investigators needs to be organized to recommend which of such diseases is the most appropriate for a clinical trial of elastase inhibitor therapy.

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§Copies of the Agenda and detailed list of participants available upon request.