Structure-Function Activity Relationships in the Inhibition of Renin

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Abstract

The development of a new technique for radioimmunoassay studies of renin inhibition by pepstatin and its analogs is described. Two pepstatin analogs containing single statine residues linked to a val-val-dipeptide were tested, and found to have no renin inhibitory activity. Two structural modifications that may produce greater inhibition of renin are proposed.

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Introduction

Control of the release of aldosterone into the circulation is of utmost importance in the long-term regulation of vascular pressure, because aldosterone stimulates sodium and water retention by promoting reabsorption of sodium from the proximal and distal tubules of the kidneys. The reabsorbtion of sodium is accompanied by a passive reabsorption of water to maintain osmolality. The amount of plasma in the circulation is thereby increased, resulting in a rise in arterial pressure.

Aldosterone is secreted from the adrenal cortex in response to prolonged drops in arterial pressure via a mechanism known as the renin-angiotensin pathway. Renin is a protolytic enzyme released from the cells of the juxtaglomerular apparatus in direct response to a fall in arterial pressure, kidney perfusion pressure, or plasma sodium. Renin catalyzes the cleavage of the tetradecapeptide renin substrate (or angiotensiogen) into a tetrapeptide and a decapeptide known as angiotensin I. Angiotensin I has no demonstrated physiological activity, but upon passage through the lungs is cleaved by a converting enzyme into a dipeptide fragment and the biologically active octapeptide angiotensin II. Angiotensin II is one of the most potent vasoconstrictors known and i.v. injection of angiotensin II into a test animal produces a dramatic but short lived increase in arterial pressure. Additionally, angiotensin II exhibits a tropic hormone effect by stimulating the release of aldosterone from the adrenal cortex (1). Aldosterone then acts to increase arterial pressure by the previously described mechanism.

It is easy to appreciate how this mechanism acts to maintain arterial pressure within acceptable limits, and it is likewise easy to appreciate that any pathological condition that interferes with this mechanism is

likely to produce substantial and serious alterations in arterial pressure. In essential hypertension, or hypertension of no known etiology, it has been suggested that the kidneys require a much higher than normal glomerular pressure in order to maintain normal excretory functions, and it seems only logical to suspect that the renin-angiotensin pathway may be involved in the elevation of arterial pressure required to produce this inappropriate increase in glomerular pressure. This is supported by the observation that plasma renin activity in humans with borderline essential hypertension are elevated (2).

Findings such as this have lent themselves to the suggestion that pharmacological manipulation of the renin-angiotensin pathway may well be beneficial in the treatment of essential hypertension. Specific agents capable of blocking the pathway at any level, used alone or in conjunction with currently available antihypertensives such as Inderal, a beta-adrenergic blocking agent, or diuretics, which promote execution of sodium, would offer the advantage of directly attacking the primary regulatory mechanism. Additionally, such a drug probably would have fewer adverse side-effects than many in use today. By replacing some drugs and being used with decreased doses of others, such agents would greatly simplify and increase the effectiveness of therapy.

There has been much research in this area during the past several years, and numerous agents have been produced which have been demonstrated to act on the renin-angiotensin pathway. These include analogs of the renin substrate that compete for renin, (3), agents which block the action of angiotensin II at its receptor sites (4), and inhibitors of the converting enzyme (5). Converting enzyme inhibitors such as Teprotide® are available for use today, and are being used to diagnose renin dependent hypertension

by monitoring changes in renin activity as the inhibitor is administered. This is based on the knowledge that renin activity actually increases in cases of dependent hypertension, despite a fall in arterial pressure as a result of the converting enzyme inhibitor. This occurs because of a feed-back loop in the pathway between angiotensin II and renin, and indicates that even inhibitors of the converting enzyme may be insufficient to effectively control hypertension.

Therefore, an agent capable of inhibiting the rate determining step - formation of angiotensin I - would be a valuable asset. In 1970 such a renin inhibitor, known as pepstatin, was isolated from streptomyces cultures (6). Pepstatin is a pentapeptide of sequence isovaleryl-L-val-L-val-statine-L-ala-statine. Positions 3 and 5 correspond to an unusual amino acid known as statine (4 amino - 3 hydroxy - 6 methylheptanoic acid) and are known to be important in producing inhibition.

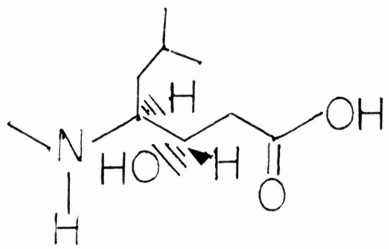

Figure 1. Statine (4S amino 3S hydroxy 6 methylheptanoic acid)

Pepstatin is a potent inhibitor, not only of renin, but also of pepsin and cathepsin D activity. It is of interest not only because of its unusual potentency, but also because it has been shown to exhibit non-competetive inhibition (7), wherein the substrate still binds to the enzyme in the presence of the inhibitor, but no reaction can occur. Such inhibition is uncommon, and study of the mechanism may provide valuable insights into enzyme inhibition.

Pepstatin, as mentioned, is unusually potent, and it has been reported that concentrations as low as .45 μg/ml inhibit the renin-substrate reaction *in vitro* by 50%. Obviously, such an inhibitor would be a valuable addition to the antihypertensive arsenal because low doses would be required for effective treatment. However, several problems hamper the use of pepstatin. As mentioned previously, it is a potent inhibitor of pepsin as well as all other carboxylproteases, making it too non-specific for use as a renin inhibitor. Additionally, the molecule is strongly hydrophopic, and maintaining a therapeutic concentration in the bloodstream would prove difficult. Accordingly, considerable research has been directed at the development of more soluble pepstatin compounds, and towards synthesis of analogs which are highly specific for renin and have little or no effect on other proteases.

Numerous analogs of pepstatin have been synthesized by the Department of Chemistry of Texas A & M University. These compounds all have in common a stereoisomeric form of statine or a close structural analog, Alastatine.

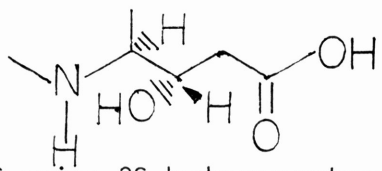

Figure 2. Alastatine (4S amino 3S hydroxypentanoic acid)

The isomers are linked covalently to a dipeptide whose structure is held constant from analog to analog.

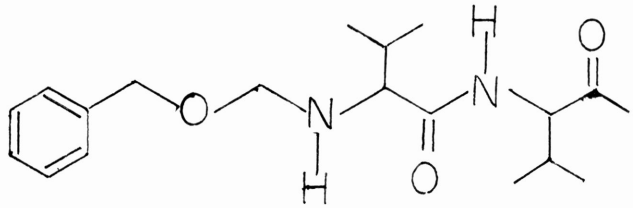

Figure 3. Valine-Valine sequence of dipeptide chain.

The analogs are tested to correlate alterations in structure with changes in biological activity. Such structure - activity relationships are useful in determining the features required for effective inhibition, and help suggest logical structural changes with an eye towards rational drug design.

In vitro testing is accomplished by allowing the endogenous renin and substrate present in a sample of dog plasma to react under conditions that favor the accumulation of angiotensin I. Renin levels are indirectly expressed as the plasma renin activity, measured in terms of nanograms of angiotensin I generated per milliter of sample per hour. By adding inhibitors in varying concentrations to the incubation mixture and moniter-ing changes in plasma renin activity, it is a simple matter to determine the effectiveness of a specific inhibitor. The angiotensin I produced during the incubation is measured by radioimmunoassay, in which the angio-tensin I reacts with a specific antibody against it. Radiolabeled angio-tensin I is added to the assay mixture, and since normal and labeled angiotensin I can compete with antibody on an equal basis, equilibrium will be reached at which a certain amount of labeled angiotensin I will be free and unbound by the antibody. Free and bound angiotensin I is then separated by physical methods and the radioactivity in each fraction measured. The activity of the bound fraction decreases with increasing levels of unlabeled angiotensin I, as the labeled form is displaced from the antibody. Reference to a standard curve relating sample activity to amounts of angio-tensin I allows an accurate determination of angiotensin I present in the sample.

This paper describes the technique of a new radioimmunoassay developed for use in studies of renin inhibition, and discusses results obtained using this assay to evaluate two possible inhibitors of renin.

Experimental Techniques

The generation of angiotensin I and its measurement by radioimmunoassay was based on an adaptation of Angiotensin I Radioimmunoassay Kit available from New England Nuclear Corporation (catalog number NEA-026).

Generation of Angiotensin I

Dog blood was collected in a chilled syringe containing EDTA to yield a concentration of 1 mg/ml. Plasma was separated in a refrigerated centrifuge at 4°C for 15 minutes at 1200 x g. Plasma was pipetted into separate plastic vials and either used at once or stored at -20°C.

One ml of chilled plasma was pipetted into a clear polystyrene tube for each inhibitor concentration to be assayed. Ten µl of aqueous Dimercaprol and 10 µl of 8-hydroxyquinoline were added to inhibit plasma angiotensinases. Two ml of .2M maleate buffer (pH 6.0) with inhibitor in solution was then added to each tube (one inhibitor concentration per tube) and mixed thoroughly.

One ml of each tube was then transferred to an identically labelled polystyrene tube, which was incubated 2 hours at 37°C±.5°. The initial tubes were kept in a 4°C ice bath for the same period. At the conclusion of the incubation the 37°C samples were returned to the ice bath and matched with their 4°C control samples.

Radioimmunoassay for Angiotensin I

All procedures were performed in an ice bath, unless otherwise stated.

A series of 18 tubes were numbered for the standard curve, along with 4 tubes for each concentration of inhibitor. This insures replication of each 37°C sample, along with its 4°C control. Five hundred µl of .1M Tris-acetate buffer (pH 7.4) was added to tubes 3 and 4 (blank

tubes).

One hundred µl of 5% Bovine Serum Albumin was added to tubes 3 and 4 (blank tubes) and 5 and 6 (zero standard). One hundred µl of the appropriate angiotensin I standard was added to tubes 7 through 18, with duplication of each standard.

One hundred µl of each 37°C incubation sample was pipetted into the appropriate tubes, and 100 µl of the matched 4°C control was added to its respective tubes. One hundred µl of radiolabeled angiotensin I (Tyrosyl-^{125}I) was added to each tube, including tubes 1 and 2 (total count). Five hundred µl of rabbit angiotensin I antisera was added to each tube except 1 through 4. Each tube was then vortexed well and allowed to equilibrate for 24 hours at 4°C. Following equilibration, 1 ml of well mixed charcoal slurry was added to all tubes except 1 and 2. The tubes were then centrifused in a refrigerated centrifuge for 20 minutes at 1200 x g. Following centrifugation, the supernatant was discarded into a waste container. The activities in the pellets were determined in a Packard Gamma Spectrophotometer for 5 minutes, and plasma renin activities for each sample determined.

Results and Discussion

The effectiveness of pepstatin in inhibiting renin was assayed in concentrations ranging from 0 to 1.0 µg/ml, and inhibitory constant (Ki) determined by use of a Dixon-Webb plot (8). A pepstatin concentration of .46 µg/ml was found to inhibit renin by 50%. This closely agrees with a previously reported value of .45 µg/ml (7), and indicates that the assay is a reliable technique for studying inhibitor effectiveness.

An analog consisting of a single statine residue linked to the peptide shown in Fig. 3 was tested for inhibitory activity, along with a

second compound composed of the structural analog Al-statine (Fig. 2) linked to an identical peptide. Assay results are summarized in Table 1.

TABLE 1

Inhibition of Renin by Pepstatin Analogs

Inhibitor Concentration	Percent Inhibition	
(μg/ml)	Alastatine	Statine
0	0	0
1	-14.6	8.5
10	0	17.1
50	-17.2	3.2
100	18.8	-9.6
500	-6.4	18.6
1000	-7.8	-.35

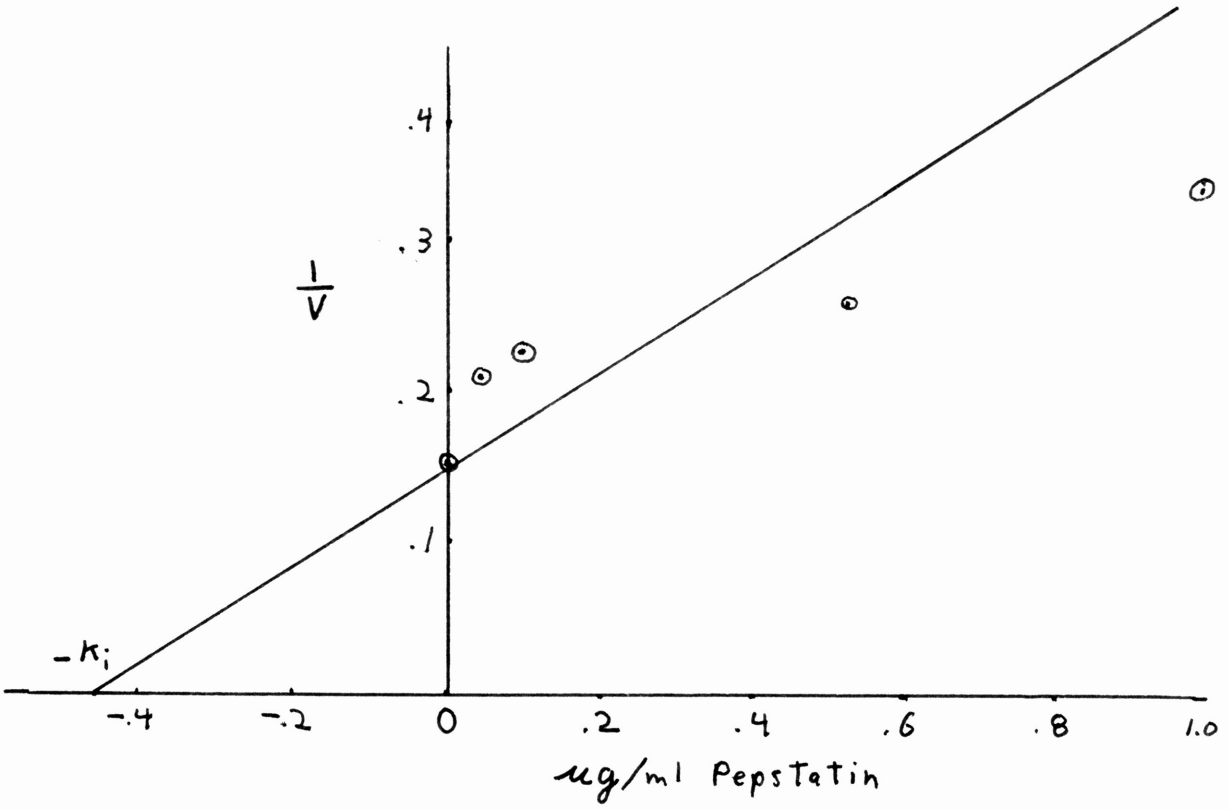

Figure 4 - Dixon-Webb plot to determine inhibitory constant of pepstatin. X-axis is concentration of pepstatin, Y-axis is reciprocal of plasma renin activity. Renin and angiotensinogen levels are assumed to be constant.

The results make it clear that neither analog showed activity in inhibiting renin, with the conclusion that a more complicated structure may be required for effective renin inhibition. This supports previously reported research (9) in which analogs of structures similar to those tested here were assayed and found to be over 450 times less active than pepstatin in inhibiting renin. Furthermore, compounds containing two statine residues were shown to be much more active than one statine analogs. Combined with the present work, this would suggest that the presence of statine in the third residue position of pepstatin may be a strict requirement for tight binding to renin. A possible approach to testing this hypothesis would be the synthesis of one statine compounds in which the statine residue is located so as to be analogous to the third residue of pepstatin. Determination of the differences in activity between such compounds and those tested here would be a valuable contribution to our knowledge of the active site. A second possibility for synthesis of inhibitors would be alterations of the peptide chain attached to the statine residue. Rather than utilize a valine-valine sequence as was done here, a leucine-leucine sequence might be adapted. The isobutyl side chain of leucine would closely approximate the isobutyl side chain of the statine residue, and may provide steric characteristics favorable to tighter inhibitor binding. Increased activity also might result from use of serine residues in the peptide, as it has been shown that the hydroxyl group present in statine is required for effective pepsin inhibition (10). Analogs in which both hydroxyl groups of pepstatin have been removed are 2000 times less effective than pepstatin at inhibiting pepsin, and the presence of the hydroxyl group may likewise be a requirement for anti-renin activity. The structures of some possible future analogs are shown in Figure 5.

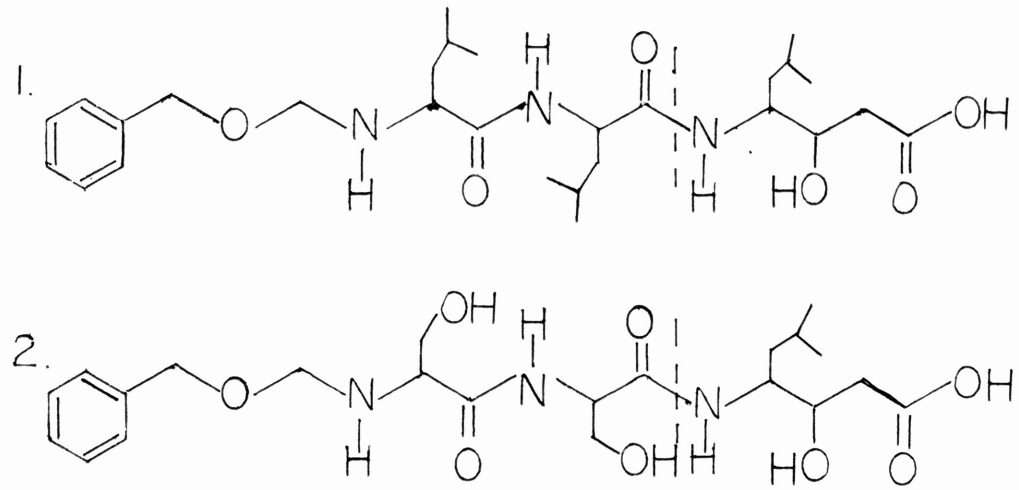

Figure 5 - Possible future pepstatin analogs. 1 has sequence leu-leu-statine; 2 has sequence ser-ser-statine. Structure to right of dotted line is statine analog.

In summary, we have developed a routine radioimmunoassay for use in studies of inhibition of renin, and found that pepstatin analogs of a sequence val-val-statine or val-val-alstatine exhibited no inhibitory activity. This confirms previously reported results and suggests that analogs more closely approximating the structure of native pepstatin will be required to produce effective inhibition of renin.

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