Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 71-77

HUMAN RENIN : A NEW CLASS OF INHIBITORS

J G Dann, D K Stammers

Department of Biochemistry
Wellcome Research Laboratories,
Langley Court, Beckenham,
Kent, BR3 3BS United Kingdom

C J Harris, R J Arrowsmith, D E Davies, G W Hardy and J A Morton

Department of Medicinal Chemistry
Wellcome Research Laboratories,
Langley Court, Beckenham,
Kent, BR3 3BS United Kingdom

Received October 18, 1985

A new class of human renin inhibitor is described, containing a novel analogue of the peptide bond. High inhibitory potency was observed for octapeptide-length substrate analogues but inhibition progressively weakened as the molecule was shortened from the amino terminal end.

The development of novel inhibitors of human renin (EC 3.4.99.19) for clinical use in antihypertensive therapy is being vigorously pursued(1).

The natural substrate for renin is the plasma protein angiotensinogen which is cleaved close to its amino-terminus by renin, releasing the decapeptide angiotensin I. This is further hydrolysed by angiotensin converting enzyme (EC 3.4.15.1) to liberate the octapeptide angiotensin II, which possesses potent vasoconstricting properties and is an important component in the regulation of blood pressure.

Aminoacid sequences for both human and animal angiotensinogens are known (2, 3) and these have been used as the basis for the design of many renin inhibitors. In particular, the octapeptide extending from the P_5 to P_3' residues of the substrate (see Fig. 1) and containing the peptide bond cleaved by renin (P_1 - P_1') has commonly been the starting point for inhibitor development.

Initially, active inhibitors were produced by the substitution of the natural P_1 and P_1' residues by other α-aminoacids; the resulting compounds had micromolar K_i

Abbreviations: TDP - tetradecapeptide fragment of equine angiotensinogen, Z - benzyloxycarbonyl, SEM - standard error of the mean.

0006-291X/86 $1.50

Subsite designation	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'
Sequence	-HIS-	-PRO-	-PHE-	-HIS-	-LEU-	-VAL-	-ILE-	-HIS-
Residue Number	6	7	8	9	10	11	12	13

Fig. 1 Partial substrate sequence of human angiotensinogen.

values (4, 5). More recently, in the search for more potent inhibitors, modifications have been made to the linkage between the P_1 and P_1' residues with the object of producing a non-hydrolysable analogue of the normal peptide bond (Fig. 2a). Reduction of the carbonyl function is an attractive approach because the resulting tetrahedral CH(OH) group in concert with the adjacent amino function could mimic part of a proposed intermediate state in the enzyme-substrate complex (6). Unfortunately the direct application of this idea is impossible because the resulting aminal function (Fig. 2b) is unstable. A number of ways are being explored in which part of the aminal function is retained and stabilised.

In one class of inhibitor (Fig. 2c) Szelke and co-workers have replaced the CH(OH) function of the aminal by a methylene group to give potent octa- to decapeptide inhibitors (7). A related strategy (Fig. 2d), which retains the desirable C_β hydroxyl group, is to replace the nitrogen atom of the aminal by a methylene group.

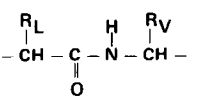
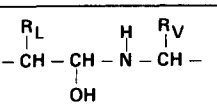
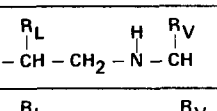
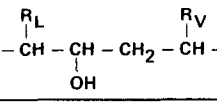
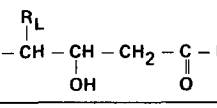
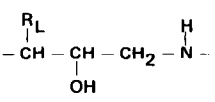
(a)		natural peptide bond
(b)		unstable aminal
(c)		reduced carbonyl analogue
(d)		hydroxyethylene analogue
(e)		statine containing
(f)		aminoalcohol analogue

Fig. 2 Chemical structure of the modified P_1 - P_1' peptide bond in renin substrate analogues. R_L and R_V represent leucyl and valyl sidechains respectively.

This has yielded a protected octapeptide inhibitor with $K_i \approx 1$ nM (8), which strongly suggests an important role for the CH(OH) function.

A second class of inhibitor incorporates the γ-aminoacid statine which occurs naturally in pepstatin, a peptidic inhibitor with specificity for acid proteases such as pepsin and renin itself (9). Statine can be substituted for leucine at the P_1 site of renin substrate analogues (Fig. 2e) to give renin inhibitors of comparable potency to those containing the reduced linkages (10, 11) in compounds of octapeptide length. In these statine-based molecules the P_1-P_1' link is lengthened to 4 atoms and the P_1-P_1' residue pair may act as a tripeptide rather than dipeptide analogue (10); it is, therefore, unlikely that the constituent aminoacids of direct substrate analogues can, simultaneously, all make similar interactions to those available to them in the substrate. A further drawback is that although statine-based inhibitors of hexapeptide length or greater are, generally, potent inhibitors of renin they are often highly lipophilic and have poor aqueous solubility unless further polar residues are added.

We report here preliminary structure-activity data for a third and new class of potent inhibitors which contain only one additional atom in the P_1 - P_1' linkage compared to the normal peptide bond (Fig. 2f). In this case a methylene group has been interposed between the CH(OH) function and the amino group of the aminal, thus providing stabilisation. This is referred to as an aminoalcohol linkage (abbreviated as .(AA). or $\underline{AA}$) and could potentially retain the favourable interactions of the tetrahedral CH(OH) group as in statine while causing less perturbation to the overall pattern of substrate-like binding. In particular, the amino function mimics more closely the position of the substrate amide function than is possible in statine. Recently, Gordon et al (12) have independently reported the successful application of a similar concept to the inhibition of metalloproteases.

Materials and Methods

Human kidney renin was purified essentially by the method of McIntyre (13) but with a preliminary step of DEAE-cellulose chromatography (as in 14) before loading on to the H77 affinity column. Renin activity was assayed by incubating the purified renin with 0.5 μM porcine tetradecapeptide renin-substrate (Sigma) for 1 hr at 37°. The assay system was buffered by 0.1 M phosphate pH 7.4 containing phenylmethyl-sulphonyl fluoride as an angiotensinase inhibitor. Liberated Angiotensin I was then quantitated by radioimmunoassay using a commerical kit (Travenol-Genentech Diagnostics) based on antibody-coated tubes.

The purified renin was examined by SDS-polyacrylamide gel electrophoresis using silver staining (14) and exhibited the reported pattern of major and minor components (13). The preparation exhibited was shown by the following activity criteria to be free from contamination by non-renin acid proteases:- (i) at pH 6.0 the ratio of activity (0.5 μM purified human angiotensinogen/0.5 μM TDP) was >10, and (ii) with 0.5 μM TDP as substrate, the ratio of activity at pH 7.4 to that at pH 6.0 was close to 1.

For measurements of IC_{50} values, inhibitor was included in the assay system at 8 - 10 concentrations around the expected IC_{50} value with 3 replicates at each concentration. Stock inhibitor concentrations were confirmed by quantitative amino-acid analysis. The activity data were fitted by weighted non-linear regression methods on an Apple microcomputer to the model

$$V_i = V_\phi / (1 + i/IC_{50}) \quad : \quad \text{where } V_\phi = \text{uninhibited activity}$$

$$V_i = \text{activity at inhibitor concentration i}$$

This expression derives readily from a model of simple competitive inhibition and permits an objective value for IC_{50} to be estimated. At least 3 separate estimates of IC_{50} were made for each inhibitor and the median estimate is quoted. The final renin concentration in the assay was ≃ 0.3 nM and therefore no correction for the depletion of free inhibitor by enzyme binding was necessary.

The inhibition type for compound BW633C was determined by varying both inhibitor and substrate concentrations within a four by four matrix. Initial rates were measured in triplicate and the means of these were fitted to different inhibition models by non-linear regression methods. The maximum TDP concentration was limited by solubility considerations.

Synthetic details for the reported compounds will be described elsewhere. All compounds were tested as ≃1:1 mixtures of isomers at the aminoalcohol hydroxyl group.

Results and Discussion

Table 1 records the structure and IC_{50} values against purified renin for this series of compounds. BW633C, an octapeptide based on the minimum substrate sequence of human angiotensinogen (cf Fig. 1), is seen to be a highly potent inhibitor

Table 1: Structures and IC_{50} Values for Aminoalcohol Inhibitors

Compound Number	Compound Structure	IC_{50} (μM)
BW455C	Z.His.Pro.Phe.His.Leu.(AA).Val.Ile.Phe.OMe	0.10
BW633C	His.Pro.Phe.His.Leu.(AA).Val.Ile.Phe.OMe	0.061
BW310C	Boc.Phe.His.Leu.(AA).Val.Ile.Phe.OMe	0.41
BW514C	Boc.Phe.His.Leu.(AA).Val.Ile.His.OMe	0.23
BW330C	Phe.His.Leu.(AA).Val.Ile.Phe.OMe	16
BW627C	Phe.His.Leu.(AA).Val.Ile.His.OMe	26
BW598C	Gly.Leu.(AA).Val.Ile.Phe.OMe	100
BW657C	His.Leu.(AA).Val.Ile.Phe.OMe	75
BW453C	Boc.Leu.(AA).Val.Ile.His.OMe	≃ 400 †
BW232C	Leu.(AA).Val.Ile.His.OMe	≃ 200 †

† Complete inhibition was not reached with these weak inhibitors.

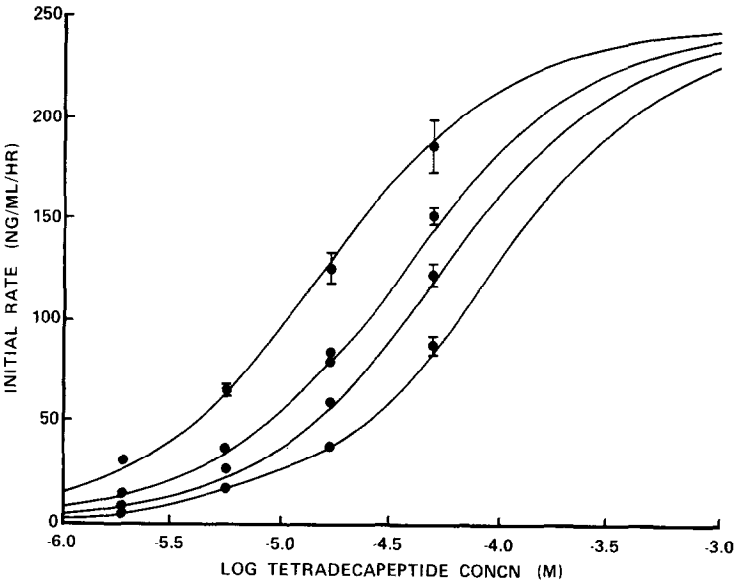


Fig. 3 The pattern of inhibition of human renin by BW633C with tetradecapeptide substrate at pH 7.4. The concentrations used in the four by four matrix were [S] = 1.9, 5.6, 16.7, 50 μM and [I] = 0, 0.05, 0.10, 0.20 μM. Experimental points are the mean ± SD of triplicate measurements. Error bars smaller than the symbol size are not shown.

of human renin at pH 7.4, with IC_{50} = 0.061 μM and was soluble to at least 0.5 mM at pH 7.4. The aminoalcohol isostere therefore confers comparable potency on octapeptide substrate analogues to that obtained with the reduced linkage (cf. H113 (8)) and statine (cf. H176 (8)), though it is significantly weaker than the hydroxyethylene linkage in H261(8). Preliminary results on the separated isomers of BW633C suggest that the isomer with the S hydroxyl group is the more active.

The kinetics of inhibition for BW633C with TDP as substrate showed it to conform closely to a competitive pattern of inhibition with K_i = 0.042 ± 0.006 μM (SEM n = 3). Fig. 3 shows the fit of observed data points and calculated lines for a typical experiment. This shows the parallel shift of the activity - [TDP] curve at increasing BW633C concentrations which is characteristic of competitive inhibition. With the natural substrate, angiotensinogen, a further mode of inhibition was revealed, additional to the competitive pattern, but the competitive K_i value was essentially unchanged at 0.053 ± 0.008 μM (SEM n = 5). These results will be reported in full elsewhere. The measured inhibition curves with TDP as substrate for all other compounds tested were fully consistent with competitive inhibition. The low substrate

concentration present in the assay (0.5 μM) relative to the K_m value (10 μM) implies that the recorded IC_{50} values will be very close to K_i values (since $K_i = IC_{50}/(1 + S/K_m)$ for simple competitive inhibition).

Initial experiments in which BW633C was administered i.v. to conscious dogs indicate that this compound has a hypotensive action in vivo.

The essential attributes of a clinically-useful renin inhibitor are high potency, oral activity and metabolic stability. Compounds such as BW633C are likely to be sufficiently potent for therapeutic use but their relatively large size and peptidic nature renders them unlikely to be transported across the gut and to be easy prey for the many proteases in the digestive tract. Smaller molecules with few natural peptide bonds are thought to be essential. Therefore the consequences of shortening BW633C from the amino terminus were investigated.

A progressive weakening of inhibitory potency was found. Protected hexapeptides as in BW310C and BW514C retained reasonable activity but deprotection caused a sudden loss of potency as in BW330C and BW627C. Replacement of His at P_3' by a Phe residue caused no significant change in binding. Further decrease in length to the tetrapeptide BW232C produced a compound with minimal inhibitory activity.

These results indicate that, as for similar statine-based and reduced analogues, $P_2 - P_3'$ oligopeptides containing the aminoalcohol linkage are not promising candidates as oral antihypertensives. However the favourable physical properties and good potency of the octapeptide-sized inhibitors make them an attractive basis for further development as diagnostic and investigational tools for the renin-angiotensin system.

Acknowledgements

We would like to thank Tony Angell, Pam Blurton, Keith Carter, Linda Russell and Chris Thomson for expert assistance and Marion Lowe for preparation of the manuscript.

References

(1) HOFBAUER, K. G. and WOOD J. W. (1985) Trends Pharmacol. Sci. April 1985, 173-177.

(2) KAGEYAMA, R., OHKUBO, H., and NAKANISHI, S. (1984) Biochemistry 23 3603-3609.

(3) OHKUBO, H., KAGEYAMA, R., UJIHARA, M., HIROSE, T., INAYAMA, S. and NAKANISHI, S. (1983) Proc. Natl. Acad. Sci. USA 80 2196-2200.

(4) POULSEN, K., BURTON, J., and HABER, E. (1973) Biochemistry 12 3877 - 3882.

(5) POULSEN, K., HABER, E. and BURTON, J. (1976) Biochim. biophys. Acta 452 533-537.

(6) MARCINISZYN, J., HARTSUCK, J.A. and TANG, J. (1976) J. Biol. Chem. 251 7088-7094.

(7) SZELKE, M., LECKIE, B., HALLETT, A., JONES, D. M., SUIERAS, J., ATRASH, B. and LEVER, A. F. (1982) Nature 299 555-557.

(8) TREE, M., BROWN, J. J., LECKIE, B. J., LEVER, A. F., MANHEM, P., MORTON, J. J., ROBERTSON, J. I. S., SZELKE, M. and WEBB, D. (1984) Biochem. Soc. Trans. 12 948-951.

(9) UMEZAWA, H., AOYAGI, T., MORISHIMA, H., MATSUZAKI, M., HAMADA, M. and TAKEUCHI, T. (1970) J. Antiobiotics 23 259 - 262.

(10) BOGER, J., LOHR, N. S., ULM, E. H., POE, M., BLAINE, E. H., FANELLI, G. M., LIN, T-Y., PAYNE, L. S., SCHORN, T. W., LAMONT, B. I., VASSIL, T. C., STABILITO, I. I. and VEBER, D. F. (1983) Nature 303 81-84.

(11) TREE, M., DONOVAN, B., GAMBLE, J., HALLETT, A., HUGHES, M., JONES, D. M., LECKIE, B., LEVER, A. F., MORTON, J. J. and SZELKE, M. (1983) J. Hypertension 1 399-403.

(12) GORDON, E. M., GODFREY, J. D., PLUSCEC, J. van LANGEN, D. and NATARAJAN, S. (1985) Biochem. Biophys. Res. Comm. 126 419-426.

(13) McINTYRE, G. D., LECKIE, B., HALLETT, A. and SZELKE, M. (1983) Biochem. J. 211 519-522.

(14) SLATER, E. E. and STROUT, H. V. (1981) J. Biol. Chem. 256 8164-8171.

(15) WRAY, W. (1981) Anal. Biochem. 118 197-203.