Volume 300, number 3, 301–304 FEBS 10900 April 1992

Slow, tight binding to human renin of some nonpeptidic renin inhibitors containing a 4-methoxymethoxypiperidinylamide at the P4 position

Herman H. Stein, Anthony K.L. Fung, Jerome Cohen, William R. Baker, Saul H. Rosenberg, Steven A. Boyd, Brian D. Dayton, Yoek Lin S.-T. Armiger, Stephen L. Condon, Robert A. Mantei and Hollis D. Kleinert

Abbott Laboratories, Cardiovascular Research Division, Abbott Park, Illinois 60064, USA

Received 6 January 1992; revised version received 19 February 1992

A series of nonpeptidic human renin inhibitors with a 4-methoxymethoxypiperidinylamide at the P4 position of the molecule exhibited slow tight binding to the enzyme. Replacement of the methoxymethoxy moiety on the piperidine ring with H, OH, methoxyethyl, propyloxy or *n*-butyl eliminated the effect. The inhibition was partially reversed by prolonged dialysis at 4°C, arguing against formation of a covalent bond in the tightened complex.

Human renin; Enzyme inhibition; Slow binding

1. INTRODUCTION

In the continuing search for inhibitors of human renin which are bioavailable by the oral route of administration, a series of nonpeptidic molecules possessing a 4-methoxymethoxypiperidinylamide group at the corresponding P4 position of angiotensinogen was synthesized [1]. During some routine studies we observed that the compounds exhibited slow tight binding properties in that the degree of inhibition increased as the preincubation time of inhibitor with renin was increased. Such behavior has not been reported previously for renin inhibitors, although it has been seen with other proteinases and peptidases such as pepsin [2] and angiotensin converting enzyme [3].

Representative members of this series have been found to be bioavailable orally in animals [4,5] and consequently may be viable candidates for the treatment of hypertension and congestive heart failure in man. It is expected that renin inhibitors will possess the same therapeutic profile as the angiotensin converting enzyme inhibitors yet will offer the potential for a lesser incidence of side effects due to the remarkable specificity of renin for its only known substrate, angiotensinogen [6]. In order to gain additional insight into the inhibitory properties of this class of compounds prior to clinical evaluation, we probed the effect of modifying the 4-methoxymethoxy substitution on the inhibition.

Correspondence address: H.H. Stein, Abbott Laboratories, D-47B, AP-10, Abbott Park, Illinois 60064, USA.

2. MATERIALS AND METHODS

2.1. *Chemical synthesis*

The synthesis of the nonpeptidic renin inhibitors has been reported [7]. In brief, the appropriate 4-substituted piperidine was reacted with L-phenyllactic acid utilizing general peptide coupling conditions. The resulting amide was alkylated with *d*-2-bromohexanoic acid to form the 2(*S*)-alkoxyacid, which was then reacted with (i) 2(*S*)-amino-1-cyclohexyl-3(*R*),4(*S*)-dihydroxy-6-methylheptane to yield compounds: **1**, **5**, **6**, **7**, **8** and **9**, or (ii) 2(*S*)-amino-1-cyclohexyl-3(*R*)-hydroxy-5(*S*)-{(dimethylamino ethoxycarbonyl)amino}-3-methylheptane to yield **4** or (iii) 2-(*S*)-amine-2-cyclohexyl-3(*S*)-hydroxy-6(*S*)-*n*-butylcarbamoyl-6-methylheptane to yield **2**. Compound **3** was prepared in a similar manner from 4-methoxymethoxy piperidine and CBZ-phenylalanine forming the nitrogen analog of the 2(*S*)-alkoxyacid described above, followed by conversion to the desired product as in (ii).

2.2. *Renin inhibition assays*

Compounds were evaluated for inhibitory activity in a purified human system at pH 6.0 as described previously [8]. The concentration of renin in the final incubation medium was 6.2×10^{-11} M based on comparison with a W.H.O. renin activity standard [9], and values of 40,000 for molecular weight and 1,170 GU/mG for specific activity [10]. The time dependency of the incubation was assessed by allowing the compound and renin to preincubate at 37°C for 1.0 and 30 min, as well as the usual 5.0 min, prior to the addition of angiotensinogen.

Studies in human plasma were performed at pH 7.4 utilizing endogenous renin and angiotensinogen [11]; the plasma was supplemented with purified human renal renin (final incubation concentration = 1.6×10^{-10} M) for the experiments in which the incubation time was 10.0 min, instead of the standard 120 min, or 240 min. The additional renin was required to increase the precision of the basal plasma renin activity measurements at that short incubation time.

The average coefficient of variation of replicated IC_{50} values in both test systems was 25%.

2.3. *Reactivation studies*

Renin [12], 60% pure protein based on specific activity, and I, both 1.0 nM, were incubated at 37°C for 2 h at pH 6.0 (50 mM maleate)

and pH 7.4 (100 mM, HEPES) in the presence of 3.0 mM EDTA, 1.45 mM phenylmethylsulfonyl fluoride, 3.4 mM 8-hydroxyquinoline and 0.1% bovine serum albumin. The enzymic activity was inhibited 100% at pH 6.0 and 85% at pH 7.4 as compared to control samples without 1. The angiotensinase inhibitors and albumin served to stabilize the renin during the inhibition step.

After the inactivation, samples were placed in ice and 0.40 ml aliquots were placed into wells of a microdialysis system 500 (Pierce) fitted with an 8,000 dalton cut-off membrane. The solutions were dialyzed at 4°C against 60 ml of the respective buffers and at various intervals 10 μl aliquots were removed and assayed for renin activity. No spontaneous reactivation occurred in undialyzed controls at either pH at 4°C.

3. RESULTS AND DISCUSSION

IC_{50} values and structures of four representative compounds, 1–4, are shown in Table I. Each of the inhibitors exhibited low nanomolar potency in the purified system at pH 6.0. Although the potency decreased in plasma at pH 7.4, the IC_{50} values were still in the 8–13 nM range.

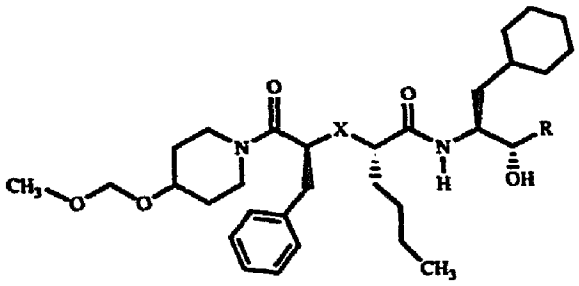
The effect of preincubation time on the percent inhibition of renin in the purified system is shown in Table II. It can be seen that each of the compounds caused progressively greater inhibition as the preincubation time was increased. The IC_{50} values decreased accordingly, by factors of 6–10, at 30 min compared to the 1.0 minute data. IC_{50} values in plasma at pH 7.4 were even more time-dependent as illustrated in Table III. The potencies increased from 11- to 31-fold, as the reaction time for the generation of angiotensin I was increased from 10 min to 240 min.

The time-dependent inhibitions observed could not be caused by an artifact arising from depletion of inhibitor as a consequence of binding to the renin. The IC_{50} value of the most potent inhibitor, 1, in the purified system was greater than the renin concentration utilized in the test by a factor of 16; in plasma, the lowest IC_{50} values were of the order of 50 times greater than the concentration of renin.

The requirement of the intact 4-methoxymethoxy group for slow binding is illustrated with the compounds in Table IV. Replacement with H, OH, $CH_3OCH_2CH_2$, $CH_3CH_2CH_2O$, or $CH_3(CH_2)_4$ in each case resulted in the loss of the property as demonstrated by the lack of increase in inhibition as the time increased; the reason for this behavior is not known. If some type of slow process were occurring with these compounds, it was completed within 1 min inasmuch as

Table I

Structure and potency of renin inhibitors



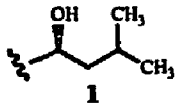
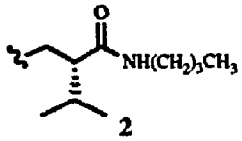
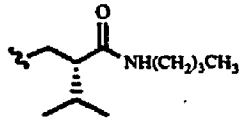
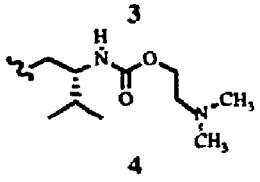
X	R	IC_{50}, nM	
		Purified	Plasma
O	 1	1.0	13
O	 2	1.5	8.2
NH	 3	1.8	8.4
O	 4	2.9	8.3

Table II

Time dependence of renin inhibition in the purified system

Compound	Purified renin inhibition (%)				IC_{50} (nM)		
	Test concentration (nM)	Renin-inhibitor preincubation time (min)			Renin-inhibitor preincubation time (min)		
		1	5	30	1	5	30
1	1.0	29	63	73	2.2	1.0	0.33
2	1.3	29	67	82	4.1	1.5	0.46
3	1.2	8	27	72	3.6	1.8	0.56
4	2.7	20	46	59	6.7	2.9	0.69

Table III

Effect of incubation time on the magnitude of renin inhibition in human plasma

Compound	IC_{50} (nM) Human plasma, pH 7.4 (min)		
	10*	120	240
1	110	13	10
2	83	8.2	6.4
3	95	8.7	3.0
4	86	9.8	6.5

*supplemented with purified human renal renin (see section 2)

Table V

Reactivation of inhibited renin by prolonged dialysis at 4°C

Reactivation time (h)	% Initial activity	
	pH 6.0	pH 7.4
0	0.0	15
76	5.5	22
120	10	34
160	16	58

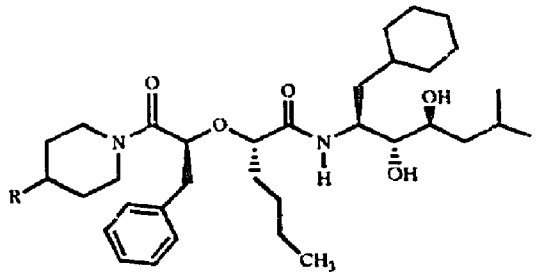
Inactivation – 2 h, 37°C, 10 nM renin and **1**. Activation – 400 ml in pierce microdialyzer, 4°C pH 6.0 (maleate) or pH 7.4 (HEPES).

the percent inhibition was unchanged (except for the *n*-butyl derivative), within experimental error, for each of the modified compounds as the preincubation time was increased from 1 min to 30 min. A possible mechanism for these observations might stem from the behavior of the 2 oxygen atoms in the methoxymethoxy side chain. The first interaction might involve a loose association of one oxygen atom with histidine-287 in the renin molecule; molecular modeling studies with inhibitor **2** and the enzyme revealed that the methoxymethoxy side chain was within hydrogen binding distance of His^{287} [13]. The second oxygen atom might be required for a stronger bonding resulting from a consequent allosteric modification of the enzyme. Studies are continuing in an effort to gain additional insight into the phenomenon.

That the renin–inhibitor complex could be dissociated by dialysis with the resultant generation of active renin and removal of the inhibitor was demonstrated with compound **1**. The results obtained, summarized in Table V, show that prolonged dialysis of the tightened complex at 4°C resulted in the regeneration of renin

Table IV

Loss of slow, tight binding property with modification of methoxymethoxy group



R	Concentration, M	Renin Inhibition, % Renin - Inhibitor Preincubation Time, Min		
		1	5	30
1 CH_3OCH_2O	1.0×10^{-9}	23	41	76
5 H	2.4×10^{-8}	49	54	49
6 HO	1.3×10^{-7}	37	37	37
7 $CH_3OCH_2CH_2$	1.8×10^{-8}	66	60	65
8 $CH_3CH_2CH_2O$	2.9×10^{-8}	72	76	80
9 $CH_3(CH_2)_2CH_2$	1.0×10^{-6}	50	45	31

capable of forming angiotensin I from antiogiotensinogen. The reactivation process was not performed at 37°C because control renin samples lost more than 50% activity before appreciable dissociation of the complex had occurred. This behavior argues against existence of a covalent bond in the complex, but rather for some type of change in conformation as discussed above.

The formation of a tightened renin–inhibitor complex may have therapeutic implication for this class of nonpeptidic inhibitors. An expected consequence would be an extended duration of action since once formed, the complex would release less renin than one subject to conventional competitive inhibition. Reversal was more easily accomplished at physiological pH, however, so it is difficult to predict how the property will be manifested in vivo. The evaluation of the overall effect awaits detailed pharmacological studies with specific compounds.

REFERENCES

[1] Portions of this work were presented at the 7th Annual Meeting of FASEB, Atlanta, April 21–25, 1991.
[2] Rich, D.H. and Sun, E.T.O. (1980) Biochem. Pharmacol. 29, 2205–2212.
[3] Goli, U.B. and Galardy, R.E. (1986) Biochemistry 25, 7136–7142.
[4] Boyd, S.A., Fung, A.K.L., Baker, W.R., Mantei, R.A., Armiger, Y.-L., Stein, H.H., Cohen, J., Egan, D.A., Barlow, J.L., Klinghofer, V., Kleinert, H.D., Verburg, K.M., Martin, D.L., Young, G.A., Polakowski, J.S., Hoffman, D.J., Garren, K.W. and Perun, T.J. (1991) Am. Chem. Soc. Meeting: Atlanta, April 14–19, MEDI-53.
[5] Kleinert, H.D., Stein, H.H., Baker, W.R., Verburg, K.M., Young, G., Fung, A., Boyd, S., Rosenberg, S., Polakowski, J., Barlow, J., Mantei, R., Klinghofer, V. and Cohen, J. (1991) FASEB J. 5, A1766.
[6] Greenlee, W.J. (1990) Med. Res. Rev. 10, 173–236.
[7] Baker, W.R., Fung, A.K.L., Kleinert, H.D., Stein, H.H., Plattner, J.P., Armiger, Y.-L., Condon, S., Cohen, J., Egan, D.A., Barlow, J.L., Verburg, K.M., Martin, D.L., Young, G.A., Polakowski, J.S., Boyd, S.A. and Perun, T.J. (1992) J. Med. Chem. (in press).
[8] Luly, J.R., Plattner, J.J., Stein, H., Yi, N., Soderquist, J., Marcotte, P.A., Kleinert, H.D. and Perun, T.J. (1987) Biochem. Biophys. Res. Commun. 143, 44–51.
[9] Bangham, D.R., Robertson, I., Robertson, J.I., Robinson, C.G. and Tree, M. (1975) Clin. Sci. Molec. Med. 48, 135s–159s.
[10] Slater, E.E. and Strout Jr., V. (1981) J. Biol. Chem. 256, 8164–8171.
[11] Plattner, J.J., Marcotte, P.A., Kleinert, H.D., Stein, H.H., Greer, J., Bolis, G., Fung, A.K.L., Bopp, B.A., Luly, J.R., Sham, H.L., Rosenberg, S.H., Dellaria, J.F., De, B., Merits, I. and Perun, T.J. (1988) J. Med. Chem. 31, 2277–2288.
[12] Stein, H., Fung, A. and Cohen, J. (1985) Fed. Proc. 44, p. 1363.
[13] Hutchins, C. et al. (in preparation).