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BINDING OF A BOLTON–HUNTER SUBSTITUTED HOMOSTATINE ANALOG TO AFFINITY-IMMOBILIZED HUMAN RENIN

JEAN-PIERRE EVENOU,* BEAT WEIDMANN, ALEXANDER HAGENBACH, RAINER METTERNICH, EMIL PFENNINGER and HERIBERT WAGNER

Preclinical Research, Sandoz Pharma Ltd, CH-4002 Basle, Switzerland

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Abstract—The binding of a Bolton–Hunter reagent substituted homostatine analog, SDZ 213-776, to human renin was investigated at pH 6.5 and 7.4. At both pH values, SDZ 213-776 bound to human renin in a reversible and saturable manner. The binding characteristics conformed to a one-site binding model. The dissociation constant K_d, obtained at equilibrium, was four-fold lower at pH 6.5 that at pH 7.4 (0.94 nM vs 3.7 nM). Under non-equilibrium conditions, only the association kinetic constant k_{+1} was affected by pH. The results of the binding assay at pH 6.5 correlated well with those obtained in enzymatic assay at the same pH.

Renin (EC 3.4.23.15) is an aspartyl protease with a stringent substrate specificity requirement and a pH optimum close to neutrality. Renin acts upon angiotensinogen, a glycoprotein synthetized in the liver, by cleaving the biologically inactive decapeptide angiotensin I (AI) at the amino-terminal. AI is, in turn, cleaved by a carboxydipeptidyl hydrolase (EC 3.4.15.1) into the potent vasoconstrictor octapeptide hormone angiotensin II (AII).

The renin angiotensin system plays an important role in the long term regulation of blood pressure. The importance of this hormonal system in the pathogenesis of essential hypertension has been, and still is, a matter of debate. However, the availability of potent angiotensin converting enzyme inhibitors and their surprisingly good efficacy in a large proportion of the hypertensive population renewed the interest in this field. The reaction of renin upon its substrate is the rate-limiting step of the whole metabolic cascade. Therefore, renin constitutes a privileged pharmacological target for the development of antihypertensive drugs [1]. In the pharmaceutical industry, the search for potent renin inhibitors has been intensely pursued for about a decade. Among several inhibition principles mimicking the transition state of the natural substrate as the cleavage site (Leu-Val), the hydroxyisostere principle (homostatine) has led to powerful compounds [2].

Human renin has proved difficult to purify, a task achieved by only a few investigators [3–7]. The small amounts of purified material available precluded the development of colorimetric or fluorimetric enzymatic assays. Currently, assay systems for the *in vitro* testing of renin inhibitors make use of a tetradecapeptide corresponding to the N-terminal sequence of human angiotensinogen [8]. The AI released by reaction of renin upon its synthetic substrate is quantitated by radioimmunoassay [9]. Recently, Cumin *et al.* [10] reported the binding of a tritiated pepstatin analog (SR 42128, Sanofi) to purified human renal renin. Separation of bound and free ligand was achieved by charcoal precipition. We describe, here, the binding of a Bolton–Hunter reagent substituted homostatin analog SDZ 213-776 (Fig. 1) to human renal renin which has been previously immobilized to a monoclonal antibody linked covalently to a rigid, magnetizable polymer matrix.

* Address correspondence to: Jean-Pierre Evenou, Building 386/725, Sandoz Pharma Ltd, CH-4002 Basle, Switzerland.

MATERIALS AND METHODS

Chemicals. SDZ 213-776 and other renin inhibitors were synthetized in our laboratories. Pure human renal renin was a kind gift of P. Corvol (INSERM U36, Paris, France). [^{125}I]-Bolton–Hunter reagent (sp. act. 2000 Ci/mmol), was purchased from Amersham. The magnetizable anti-human renin monoclonal antibody (3E8) and the IRMA kit for the determination of active renin in human plasma (79895) were from Pasteur Diagnostics. Aprotinin (Trasylol®, 20,000 K I.U./mL) was from Bayer. Bovine serum albumin (Cohn fraction V) was from Fluka. Human tetradecapeptide renin substrate (Asp^{1}-Asn^{14}) and H.142 were from Bachem. All other chemicals were of analytical grade.

Crude kidney homogenate. Human kidneys were obtained at autopsy and were kept frozen at −70°. After thawing on ice, cortices were dissected, minced and homogenized in 50 mM Imidazole–HCl buffer pH 7.2 containing 0.5 mM *N*-ethylmaleimide, 5 mM Na_2-EDTA, 0.1 mM diisopropyl fluorophosphate and 20,000 K.I.U./mL Aprotinin, for 2 min at 4° using a Polytron homogenizer. The homogenates were quickly frozen in dry-ice/acetone. The cycle of thawing and freezing was repeated twice. The homogenates were finally centrifuged at 100,000 *g* for 30 min at 4°. The supernatants were pooled and kept frozen in suitable aliquots at −20° until use.

[^{125}I]*SDZ* 213-776. Composition of iodination mixture was as follows: 1 mCi of Bolton–Hunter reagent,

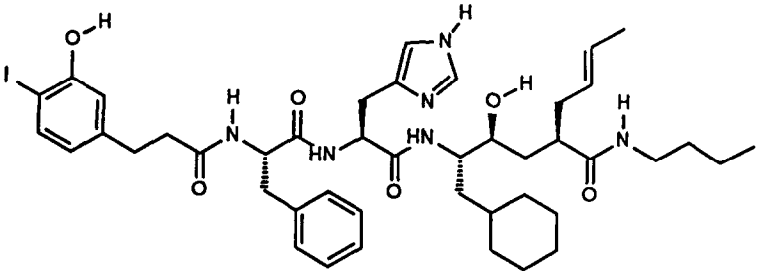

Fig. 1. Chemical structure of SDZ 213-776.

5 μmoles of unsubstituted peptide and 15 μmoles of *N*-ethylmorpholine in a total volume of 250 μL dimethylformamide. The mixture was divided into small aliquots which were incubated separately for 4 hr at 37°. The splitting of the original mixture was found necessary in order to achieve a sufficient iodination yield. Purification of the ligand was performed by HPLC on a Brownlee (Brownlee Labs) reversed phase cartridge RP-18, 220 × 4.6 mm, at a flow rate of 1 mL/min using 0.25 N triethylamine-phosphate buffer pH 3.0/acetonitrile (50:50) as mobile phase. UV-absorbance at 250 nm and radioactivity were recorded throughout. Fractions of 0.5 mL were collected. The fraction containing the highest radioactivity was immediately neutralized by addition of 150 μL of 0.5 M phosphate buffer pH 7.4. Fifty μL of a 10% Tween 20 solution and 100 μL of an ascorbic acid solution (10 mg/mL) were also added in order to minimize loss of ligand due to oxidation or unspecific adsorption. The ligand solution was divided into suitable aliquots and kept frozen at −20° under nitrogen.

Active renin concentration. The measurement of active renin concentration using an immunoradiometric procedure was performed according to kit specifications. Standards consisted of primate renin and ranged between 10 and 640 pg/mL.

Enzymatic assay. Pure human renal renin (55 pM) was mixed together with human tetradecaptide (11.4 μM) in a total volume of 250 μL of 0.5 M citrate–phosphate buffer pH 6.5 containing 0.1% BSA. The mixture was incubated at 37° for 30 min. The enzymatic reaction was stopped by boiling the samples at 95° for 10 min. The generation of AI was quantitated by radioimmunoassay using a commercial kit (CIS).

Binding assay. One hundred μL of kidney supernatant (renin concentration = 0.56 nM as determined by IRMA) were mixed together with 100 μL of magnetizable antibody suspension. The mixture was incubated for 2 hr at 37° under constant and thorough shaking. Incubation was stopped by addition of 1 mL of chilled 50 mM phosphate buffer pH 7.4 containing 0.1% BSA and 0.01% Tween 20 (assay buffer). The tubes were allowed to stand for 2 min. Magnetic separation of the pellet was achieved by placing the tubes onto a magnetic rack (Serono Diagnostics) for 2 min. Supernatants were discarded. Care was taken at this step to remove any remaining liquid drops. One hundred μL of ligand (4 nM, 50,000 cpm) was added and the mixture allowed to incubate for 60 min at 37° under constant and thorough shaking. Unspecific binding was determined by incubating the ligand with a large excess (1000-fold) of the compound H.142 [11]. Separation of bound and free radioactivity was achieved by washing the pellet three times with 1 mL of ice-cold 50 mM phosphate buffer pH 7.4 containing 0.5% Tween 20 followed by magnetic separation. Radioactivity bound to the pellet was measured in a multiwell, gamma-counter at 80% counting efficiency.

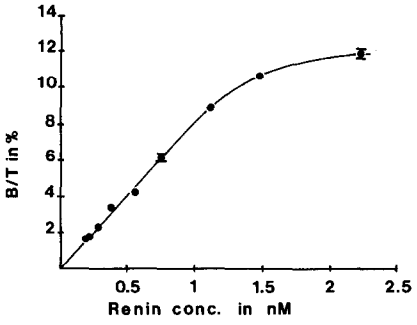

Fig. 2. Specific binding of [^{125}I]SDZ 213-776 at various renin concentrations. Enzyme concentrations were estimated independently by immunoradiometric assay. Points are means ± SE of triplicates.

Competition binding assay. Competitors (10^{-10}–10^{-7} M in assay buffer) were added to standard incubation mixture. K_d values were calculated by non-linear regression analysis of displacement binding curves according to a one-site binding model. The computer program was kindly provided by Dr H. J. Tobler.

RESULTS

Iodination procedure

Under the experimental conditions described above, the yield of iodinated product was typically over 70%. During HPLC separation, [^{125}I]SDZ 213-776 eluted as a single, symmetrical peak.

Binding capacity of anti-renin monoclonal antibody

Monoclonal antibodies (MAB) to human renin were originally developed by Galen *et al.* [12]. The MAB with code 3E8 did not inhibit the enzymatic activity of renin *in vitro*, indicating that the binding

Table 1. Enzyme and species specificity of SDZ 213-776

Enzyme	IC_{50} (M)	Selectivity (fold)
Human renin (pH 6.5)	7×10^{-10}	1
Mouse renin* (submaxillary gland)	8.5×10^{-9}	12.1
Cathepsin D†	8.5×10^{-6}	12,140
Pepsin‡	1×10^{-5}	14,300
Angiotensin converting enzyme§	$>1 \times 10^{-4}$	>143,000

* Assay was performed at pH 6.5 using pure MSG renin according to K. Murakami *et al.* (*Anal Biochem* **110**: 232–239, 1981).

† Assay was performed at pH 3.8 using semi-purified cathepsin D from bovine spleen according to H. R. Williams and T . T. Lin (*Biochim Biophys Acta* **250**: 603–607, 1971).

‡ Assay was performed at pH 2.0 using semi-purified pepsin from porcine stomach mucosa according to previous reference.

§ Assay was performed at pH 8.3 using semi-purified ACE from rabbit lung according to M. S. Rohrbach (*Anal Biochem* **84**: 272–276, 1978).

epitope is not situated in the vicinity of the enzyme active site. Using iodinated renin, the association constant, K_a, of MAB 3E8 was found to be 1.2×10^9 M^{-1}. We investigated the binding capacity of MAB 3E8 for human renal renin by incubating 100 μL of a serial dilution of kidney supernatant in the presence of 100 μL of magnetizable antibody suspension (Fig. 2). The binding was found linear up to a renin concentration of 1 nM. For enzyme concentrations above 2 nM, saturation was achieved. A renin concentration of 0.56 nM was used for further investigations.

Ligand stability

The possibility that, during the first incubation step, proteases with broad specificity could bind non-specifically to the matrix and later degrade the ligand during binding assay, was investigated. For this purpose, an aliquot of the native ligand solution and an aliquot of the mixture following 1 hr incubation at 37° in the presence of antibody-immobilized renin, were run under similar conditions on TLC silica plates using the following system: CH_2Cl_2/CH_3OH + 1% anhydrous NH_4OH (95:5). Following incubation at 37°, a single peak, superimposable with the peak of the native fraction, was obtained indicating ligand stability under our assay conditions.

Enzyme and species specificity of SDZ 213-776

The inhibitory potency of our compound was

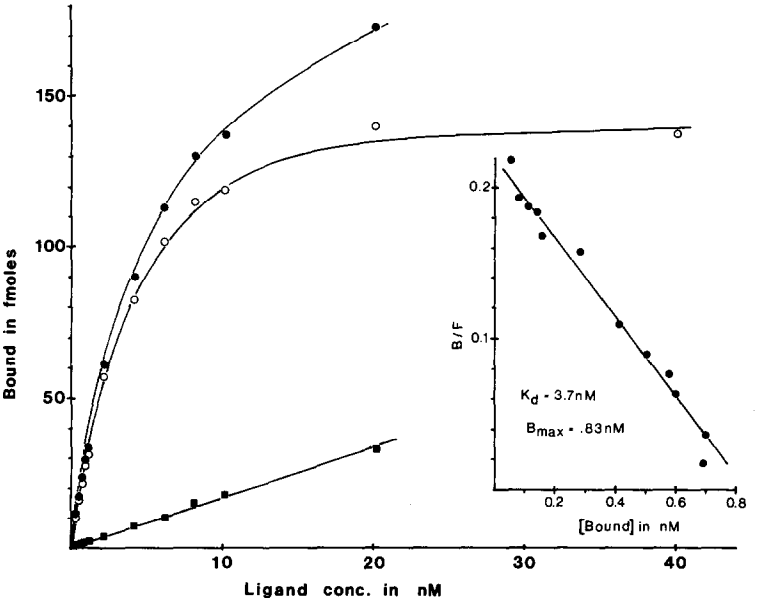

Fig. 3. Saturation experiment. Total (●), specific (○) and non-specific (■) binding of [^{125}I]SDZ 213-776 at various ligand concentrations. Points are means of triplicates. Scatchard plot is shown as insert.

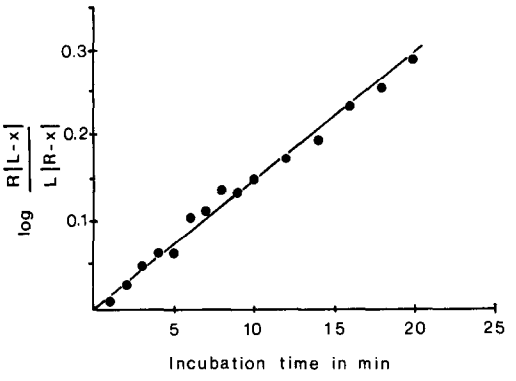

Fig. 4. Association study. Plot of logarithmically transformed data vs association reaction time. The incubation was performed batchwise. Following addition of reactants, small aliquots were withdrawn at regular time intervals and processed as described in Materials and Methods.

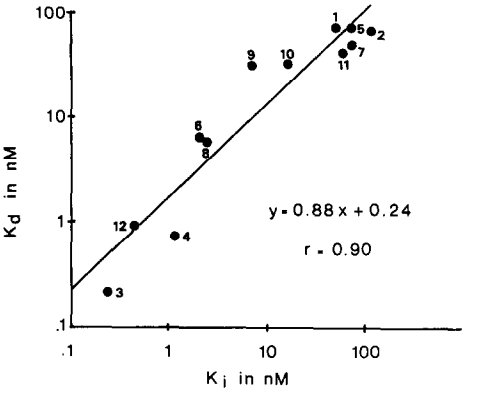

Fig. 6. Correlation between K_d values obtained from competition binding assays at pH 6.5 and K_i values obtained from enzymatic assays at the same pH.

assayed against mouse submaxillary gland renin, related aspartyl proteases and angiotensin converting enzyme according to standard procedures. The results are depicted in Table 1. SDZ 213-776 was found to be highly renin specific.

Saturation experiment

Increasing concentrations of ligand (0.29–40 nM) were incubated in the presence of a fixed concentration of renin (0.56 nM). The results, as shown in Fig. 3, indicate that the peptide bound to human renin in a saturable manner. Non-specific binding was measured by incubating the ligand in the presence of a large excess of the hydrophilic renin inhibitor H.142. The values of non-specific binding obtained were similar to those obtained in the absence of enzyme, indicating that the ligand was specifically displaced from the renin molecule by H.142. Scatchard plot was typical of a single binding site. The calculated dissociation constant (K_d) and B_{max} were 3.7 nM and 0.83 nM, respectively.

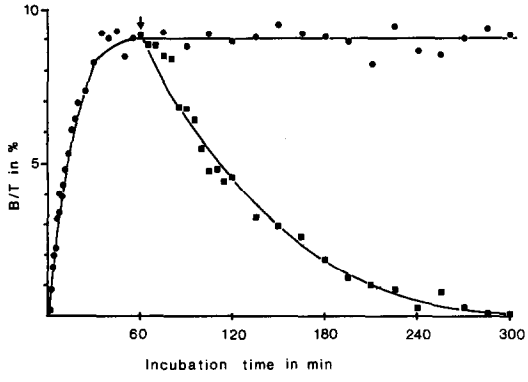

Fig. 5. Association/dissociation studies. The association reaction (●) was performed batchwise. Dissociation (■) was induced at arrow by addition of a large excess of H.142. At regular time intervals, small aliquots were withdrawn and processed as described in Materials and Methods.

Determination of kinetic association constant (k_{+1})

[^{125}I]SDZ 213-776 (4 nM) was incubated with renin (0.56 nM) for periods ranging from 1 min to 5 hr. Corrections for non-specific binding were done. Analysis of data was performed on the first 20 min of the association reaction, where the following bimolecular reaction can be assumed:

$$R + L \xrightarrow{k+1} RL.$$

Transformation of the data was performed according to the following equation:

$$\log \frac{R[L-x]}{L[R-x]} = k_{+1} \frac{[L-R]}{2.303} \cdot t$$

where R = concentration of receptor at time 0, L = concentration of ligand at time 0, x = concentration of ligand–receptor complex at time t. Linear regression analysis plot shown in Fig. 4 yielded a value of 8.6×10^6 M^{-1} min^{-1} for the association parameter, k_{+1}.

Determination of kinetic dissociation constant (k_{-1})

[^{125}I]SDZ 213-776 (4 nM) was incubated with renin (0.56 nM) for 60 min in order to reach equilibrium. Dissociation was induced by adding the compound H.142 at a concentration of 4 μM. The dissociation reaction was followed during 4 hr (Fig. 5). Analysis of data was performed according to the following equation:

$$[RL] = [RL]_0 \, e^{-kt}$$

where $[RL]_0$ and $[RL]$ represent, respectively, the concentrations of the ligand–receptor complex at time 0 and at time t of the dissociation reaction. Linear regression analysis plot of logarithmically transformed data yielded a value of 0.014 min^{-1} for the kinetic dissociation parameter, k_{-1} ($y = -0.014 x + 8.48$, $r = 0.98$).

Correlation between competition binding assay and enzymatic assay

The inhibitory activities of several renin inhibitors listed in Table 2, were investigated at pH 6.5 using

Table 2. Dissociation and inhibitory constants of renin inhibitors possessing different inhibitory principles. Each value is the mean of two or three separate determinations

Substance	Structure*	Binding assay K_d (nM) pH 7.4	Binding assay K_d (nM) pH 6.5	Enzyme assay K_i (nM) pH 6.5	Plasma renin inhibition IC_{50} (nM)
1 214-553	Boc-Phe-Nle-Cha(OH)CH_2CH(OH)CH_2O-i-Pr	550	71.0	48.4	ND
2 214-549	AcGlycOiBu-Phe-Nle-SO_2-Chatin-N(Me)$_2$	310	65.0	116	ND
3 214-423	Boc-Gly-4egCO-Phe-Nle-Cha-OH-Bly-NH-*n*-Bu	0.46	0.22	0.23	1.1
4 214-422	Chol-4egCO-Phe-Nle-Cha-OH-Bly-NH-*n*-Bu	1.50	0.72	1.10	13
5 214-419	Boc-Phe-Nle-Cha-OH-$CH_2CH_2PO(OEt)_2$	450	71.0	71.0	ND
6 214-285	Suc-Phe-His-Cha-OH-Bly-NH-*n*-Bu	26.0	6.30	2.0	10
7 214-284	TocOSuc-Phe-His-Cha-OH-Bly-NH-*n*-Bu	260	48.0	71.0	ND
8 214-283	Boc-Phe-Bly-ChaCF_2CF_2CO-Leu-2-Pic	21.0	5.70	2.39	60
9 214-282	Boc-Phe-Bly-Cha(OH)CF_2CF_2CO-Leu-2-Pic	140	32.0	7.10	ND
10 213-853	Dinac-Leu-Cha(OH)CH_2NHCONH-i-Pr	380	32.0	16.1	ND
11 213-842	Dinac-His-Cha(OH)CH_2NHCONH-i-Pr	290	40.0	58.1	ND
12 213-776	BoH-Phe-His-Cha-OH-Bly-NH-*n*-Bu	3.70	0.94	0.43	19

* Abbreviations used: Cha, β-cyclo-hexylalanine; Bly, 2-butenylglycine; *n*-Bu, *n*-butyl; Boc, tert-butyloxycarbonyl; i-Pr, isopropyl; Ac, acetyl; Glyc, (α)-D-glycosyl; i-Bu, isobutyl; SO_2-Chatin, 5-cyclo-hexyl-4-amino-3-hydroxypentane sulfonic acid; 4egCO, O$(CH_2$–$CH_2O)_4$–CO; Chol, cholic acid; Suc, succinyl; Toc, α-tocopheryl; Pic, 2-aminomethylpyridine; Dinac, di(1-naphtylmethyl)acetyl; BoH, *N*-succinimidyl 3-(4-hydroxy-5-iodophenyl)propionyl.

the enzymatic assay described above. K_i values were estimated according to the following formula [13]:

$$K_i = \frac{IC_{50}}{1 + S/K_m}.$$

A K_m value of 20.7 μM was used for the reaction of human tetradecapeptide (Asp^1-Asn^{14}) with pure renin according to Cumin *et al.* [19]. A poor correlation was found between the K_i values measured at pH 6.5 by the enzymatic assay and the K_d values obtained from competition binding assay at pH 7.4. The discrepancies observed might have been due to difference in pH resulting in different charge distributions at the enzyme active site. The binding of [^{125}I]SDZ 213-776 to human renin was therefore investigated at pH 6.5. Following parameters were calculated: $K_d = 0.94$ nM, $B_{max} = 0.63$ nM, $k_{+1} = 2.4 \times 10^7\ M^{-1}\ min^{-1}$, $k_{-1} = 0.02\ min^{-1}$. Competition binding assays with all the substances listed in Table 2 were repeated at pH 6.5. The calculated K_d values correlated well with the K_i values as shown in Fig. 6.

DISCUSSION

The binding of a potent renin inhibitor (SDZ 213-776) to human renal renin was investigated and the potential of this method for the *in vitro* screening of renin inhibitors was evaluated.

The binding of SDZ 213-776 to human renin was reversible and saturable, with a single type of ligand–receptor interaction. The association kinetic parameter (k_{+1}) was strongly pH-dependent, whereas the dissociation kinetic constant (k_{-1}) remained unaffected. Similar findings were reported by Cumin *et al.* [10]. Rate constants for bimolecular collisions are dependent on the size (and shape) of both molecules and on diffusion. According to Gutfreund [14], the values of k_{+1} obtained in transient kinetic studies for the interactions of enzymes with ligands average $1–2 \times 10^9\ M^{-1}\ min^{-1}$. In our studies the calculated value for the association parameter at pH 6.5 was about two orders of magnitude smaller. The discrepancy might be due, either to steric hindrance or to the formation of an intermediary collision complex. Interestingly, the first 20 min of the association reaction of SDZ 213-776 and renin were adequately described by a bimolecular reaction model. The pH-dependence of the association constant demonstrates the importance of the protonation status of one or more residues situated at the enzyme active site for the binding process.

It is noteworthy that the binding of renin to the monoclonal antibody corresponds to a pseudo-immobilization governed by the mass-action law.

However, due to the high affinity of MAB 3E8 for human renin and the relatively low diffusion rate of the renin molecule (MW ≈ 40 kD), a strong interference of this binding equilibrium with the binding of the ligand to renin is unlikely. In our hands, the binding properties of the ligand to affinity-immobilized renin, kept as a suspension at 4°, remained unaltered for up to 6 months.

The enzymatic assay was performed at pH 6.5, a value which corresponds to the pH-optimum for the reaction of pure human renin upon synthetic tetradecapeptide substrate [9]. The K_d values calculated from competition binding assay at this pH correlated well with the K_i values obtained from the enzymatic assay, indicating that our binding protocol represents a useful alternative to previous methods for the *in vitro* testing of renin inhibitors. Among the substances listed in Table 2, those which displayed K_d values in the nanomolar range were further investigated in primate models. Prior to *in vivo* evaluation, such compounds were assayed *in vitro*, for their inhibitory potency toward human plasma renin using the Antibody Trapping Assay [15]. As can be seen, K_d values obtained at pH 7.4 offer the best predictability to *in vivo* situation. For this reason, we are now routinely performing binding assays at physiological pH.

In our binding protocol, the availability of a pure human renin source is no longer a prerequisite. The use of anti-renin monoclonal antibody linked to a magnetizable matrix is easier for the purification and insolubilization of the enzyme molecule, as well as for the separation of bound and free ligand.

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REFERENCES

1. Greenlee WJ, Renin inhibitors. *Pharmaceut Res* **4**: 364–374, 1987.
2. Szelke M, Tree M, Leckie BJ, Jones DM, Atrash B, Beattie S, Donovan B, Hallet A, Hughes M, Lever AF, Morton JJ and Sueiras-Diaz J, A transition-state analog inhibitor of human renin (H.261): test *in vitro* and a comparison with Captopril in the anaesthetized baboon. *J Hypertension* **3**: 13–18, 1985.
3. Galen FX, Devaux C, Guyenne T, Menard J and Corvol P, Multiple forms of human renin. *J Biol Chem* **254**: 4848–4855, 1979.
4. Yokosawa H, Holladay LA, Inagami T, Haas E and Murakami K, Human renal renin. *J Biol Chem* **255**: 3498–3502, 1980.
5. Slater EE and Strout HV, Pure human renin. *J Biol Chem* **256**: 8164–8171, 1981.
6. McIntyre GD, Leckie B, Hallet A and Szelke M, Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77. *Biochem J* **211**: 519–522, 1983.
7. Shinagawa T, Do YS, Tam H and Hsueh WA, Complete purification of human renal renin and sequence of the amino terminus. *Biochem Biophys Res Commun* **139**: 446–454, 1986.
8. Tewksbury DA, Dart RA and Travis J, The amino terminal amino acid sequence of human angiotensinogen. *Biochem Biophys Res Commun* **99**: 1311–1315, 1981.
9. Cumin F, Le-Nguyen D, Castro B, Menard J and Corvol P, Comparative enzymatic studies of human renin acting on pure natural or synthetic substrates. *Biochim Biophys Acta* **913**: 10–19, 1987.
10. Cumin F, Nisato D, Gagnol JP and Corvol P, Binding of a tritiated pepstatin analog to human renin. *J Cardiovasc Pharmacol* **10** (Suppl. 7): S102–S104, 1987.
11. Szelke M, Leckie B, Hallet A, Jones DM, Sueires J, Atrash B and Lever AF, Potent inhibitors of human renin. *Nature* **299**: 555–557, 1982.
12. Galen FX, Devaux C, Atlas S, Guyenne T, Menard J, Corvol P, Simon D, Cazaubon C, Richer P, Badouaille G, Richaud JP, Gros P and Pau B, New monoclonal antibodies directed against human renin. *J Clin Invest* **74**: 723–735, 1984.
13. Cheng YC and Prusoff WH, Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
14. Gutfreund H, Kinetic analysis of the properties and reactions of enzymes. *Prog Biophys Mol Biol* **29**: 161–195, 1975.
15. Poulsen K and Jørgensen J, An easy radioimmunological microassay of renin activity, concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody. *J Clin Endocrinol Metab* **39**: 816–825, 1974.