Mechanism of Inhibition of Human Renin by Monoclonal Antibodies

MARC M. DE GASPARO, JEANETTE M. WOOD, AND CHRISTOPH H. HEUSSE

With the technical assistance of Michèle Mele

SUMMARY The mechanism by which monoclonal antibodies directed against human renin (R3-36-16 and R3-47-10) inhibit renin activity was investigated using various substrates. Both antibodies acted as potent inhibitors of human renin activity when human angiotensinogen was used as a substrate. However, their effects differed clearly in the presence of synthetic tetradecapeptide. When low concentrations of tetradecapeptide were used as substrate, renin activity was only partially inhibited by R3-47-10, whereas it was stimulated by R3-36-16. At higher synthetic substrate concentrations, both antibodies stimulated angiotensin I production. This effect was independent of the pH. Both antibodies exerted their effects in the presence of CGP 29287, a peptidic transition-state competitive renin inhibitor, indicating that their binding sites differed from that of CGP 29287. In combination, the stimulatory effect of R3-36-16 was not blocked by R3-47-10, but the inhibition produced by R3-47-10 was reversed by R3-36-16. Both antibodies may prevent the large natural substrate angiotensinogen from entering the enzymatic cleft by steric hindrance. At a low substrate concentration, R3-47-10 may also partially hinder the access of synthetic tetradecapeptide into the active cleft by steric hindrance. In contrast, the stimulating effect of both antibodies may be due to a conformational change in the renin molecule, allowing an increased access of tetradecapeptide or a more rapid release of the product from the enzymatic cleft. (Hypertension 11: 209-216, 1988)

KEY WORDS • angiotensinogen • tetradecapeptide • renin inhibitor • binding site • enzyme kinetics • dose-response analysis

MONOCLONAL antibodies may exert different effects on the function of the target molecule, depending on the particular epitope to which they bind.1-4 For example, monoclonal antibodies directed against human renin bind specifically to renin with varying affinities, but not all of them inhibit the activity of the enzyme.4 Very little is known about the epitopes of these antibodies. Recently, synthetic peptides corresponding to different regions of the renin molecule were tested for their binding to various antibodies, and several renin epitopes were identified.5-6

The monoclonal antibodies R3-36-16 and R3-47-10 both bind to renin and are the most potent inhibitors so far described.4 7 8 They are specific for human renin and do not bind to other aspartic proteinases such as human cathepsin D, pepsin, or gastricsin.4 However, neither of them distinguishes active from inactive renin, suggesting that they probably bind outside the active cleft. As R3-36-16 and R3-47-10 cross-inhibited each other in a binding assay,4 they were assumed to bind to a common area. The present study was undertaken to elucidate the mechanism by which R3-36-16 and R3-47-10 inhibit renin. Their inhibitory action was analyzed by examining their effects of two different substrates, angiotensinogen and tetradecapeptide (TDP). The effects of a peptidic transition-state inhibitor that inhibits renin by binding within the active cleft were also studied. In addition, the interaction between the antibodies and the transition-state renin inhibitor was investigated.

Materials and Methods

Purification of Human Renin

An extract of human kidney renin was prepared according to a modification of the method of Haas et al.9 Briefly, frozen kidneys were ground, extracted with water, and filtered. The filtrate was acidified to pH 2.8 with 5 N sulfuric acid and precipitated with 0.8 M sodium chloride. The supernatant was precipitated with ammonium sulfate (1-2.3 M). The pellet was resuspended in water, dialyzed against water, and then

From the Research Department, Pharmaceuticals Division, CIBA-Geigy Limited, Basel, Switzerland.

Address for reprints: M. de Gasparo, CIBA-Geigy, K 125.10.15, Postfach, CH-4002 Basel, Switzerland.

Received April 2, 1987; accepted October 23, 1987.

209
centrifuged. The supernatant yielded renin at a concentration of 1.5 Goldblatt units (GU)/ml and with a specific activity of 0.2 GU/mg protein. This extract was further purified by immunoabsorption on a monoclonal antibody Sepharose 4B column. The final specific activity of renin was 150 to 200 GU/mg protein.

### Angiotensinogen and Tetradecapeptide Substrates

Angiotensinogen was extracted from human plasma according to a modification of the procedures of Tewksbury and Dart and Kokubu et al. All procedures were performed at 4°C. To remove renin, plasma collected on EDTA was mixed with a polyclonal antibody against renin coupled to a Sepharose 4B gel. After 14 hours, the gel was removed by filtration. Angiotensinogen was precipitated from the plasma with ammonium sulfate (1.5–2.4 M). Albumin was removed by chromatography on an Affigel column eluted with Tris buffer.

The fractions containing angiotensinogen were further purified, first on a diethylaminoethyl Sephacel column (Pharmacia, Uppsala, Sweden) and then on a hydroxypatite column. During this procedure, angiotensinogen was purified 900-fold. The total recovery was 27%.

The TDP renin substrates contained either the hog (Bachem) or human (Peninsula) amino acid sequence. All substrates were diluted to the appropriate concentration in deionized water containing 1% human serum albumin (Swiss Red Cross) and adjusted to pH 7.8 with 0.1 N sodium hydroxide.

### Enzymatic Assay

The purified human kidney renin (25 pM) was incubated with either renin-free human angiotensinogen or human (0.5–6 μM) or hog (2.5–500 μM) TDP substrates in 0.1 M phosphate buffer containing 1% human serum albumin, pH 7.0. The wide range of hog TDP concentrations was required to estimate the kinetic constants accurately. Higher concentrations of human TDP could not be used because of cross-reaction with the antibodies in the angiotensin I radioimmunossay. The reaction was performed at 37°C and the incubation was stopped after 1 hour by adding an excess of cold buffer and transferring the tube onto ice. The angiotensin I generated was measured by radioimmunoassay as described previously. The monoclonal antibodies (added alone or together) or CGP 29287 (CIBA-Geigy), or both were incubated with renin and substrate. In some experiments, a range of antibody concentrations were incubated at a constant substrate concentration, thereby obtaining a value for the concentration of antibody required to inhibit velocity by 50% (ED50). In other experiments, the second antibody was added after preincubation for 1 hour with the first antibody and the angiotensin I production was measured over the 2-hour period. For each substrate concentration, a tube without renin was added to measure any nonspecific production of angiotensin I. There was no interference between the monoclonal antibodies against renin and the angiotensin I antibodies used in the radioimmunossay. For comparison, some experiments were also performed with Medical Research Council (MRC) renin (68/356) (London, UK) and recombinant renin (gift from Dr. V. J. Dzau, Boston, MA, USA).

### Monoclonal Antibodies

Monoclonal antibodies were prepared by immunization of BALB/c mice with a purified preparation of human kidney renin and subsequent fusion of the mouse spleen cells with a nonsecretor myeloma cell line. A detailed description of the production and characterization of the highly purified antibodies has been given in a separate publication.

Briefly, the two antibodies selected for this study, R3-36-16 and R3-47-10 bind (binding capacity, 50%; 6.2 × 10⁻¹¹ and 7.0 × 10⁻¹¹ mol/L) and inhibit (IC50, 1.3 × 10⁻¹⁰ and 2.0 × 10⁻¹¹ mol/L) renin to a similar extent. Moreover, they cross-inhibit each other in the binding assay.

### CGP 29287

The CGP 29287, which was synthesized in the laboratories of CIBA-Geigy (Drs. B. Riniker and P. Bühlmayer), is an analogue of the amino acid sequence of human angiotensinogen. It contains statine, the unusual amino acid from pepstatin, as a transition state mimic and has C-terminal protecting groups. It is a potent and specific competitive inhibitor of primate renin.

### Analysis of the Data

The results are expressed as means ± SEM. The data were statistically evaluated using Dunnett's test. Significance was set at a p level below 0.05. The kinetic constants of the enzymatic reaction were determined for each curve by applying the Michaelis-Menten equation to the raw data with the help of a computer. As the Michaelis-Menten equation may be transformed into a sigmoid by expressing the substrate concentration logarithmically, the Allfit four-parameter logistic dose-response analysis program of De Lean et al. written in Applesoft BASIC (Apple Computer, Cupertino, CA, USA) by M. H. Teicher (Harvard Medical School, Boston, MA, USA) was also used on the mean values. This program can fit a number of curves simultaneously and allows analysis of a group of sigmoidal dose-response curves. The main advantage of the Allfit program is that the hypothesis that some curves share common parameters can be tested by forcing the curves to share these parameters and by verifying that the effect of these constraints on the goodness of fit is minimal. The Ks of the antibodies was approximated from the ED50 using the equation of Cheng and Prusoff.

### Results

#### Influence of the Renin Substrate

The effects of R3-47-10 and R3-36-16 were first tested in the presence of a fixed concentration of hu-
INHIBITION OF RENIN BY MONOCLONAL ANTIBODIES
Gasparo et al.

R3-47-10

% of the control value

7 40 70 100 300 [Ab] [M]

R3-36-16

% of the control value

7 40 70 100 300 [Ab] [M]

FIGURE 1. Effect of increasing concentrations of R3-47-10 (upper panel) and R3-36-16 (lower panel) on angiotensin I production from 2.5 μM hog (*) or human (*) tetradecapeptide and 0.4 μM human angiotensinogen (*). Results are expressed as a percentage of the control value (means ± SEM; n = 12 for each concentration).

man angiotensinogen (0.4 μM) or hog or human TDP (2.5 μM; Figure 1). Both antibodies inhibited angiotensin I production from angiotensinogen in a dose-dependent manner, reaching total inhibition at an antibody concentration of 0.7 nM. The Kd values were 41.7 ± 3.2 (n = 5) and 47.6 ± 6.3 pM (n = 4) for R3-47-10 and R3-36-16, respectively. With hog or human TDP as substrate, the inhibitory effect of R3-47-10 (0.7 nM) was only partial (50%). The Kd values of R3-47-10 for renin were 49 ± 5.5 (hog TDP; n = 7) and 46 ± 5.4 pM (human TDP; n = 7). By contrast, R3-36-16 (0.7 nM) significantly stimulated renin activity, increasing angiotensin I production from human and hog TDP by 1.8-fold (maximum velocity of the reaction [Vmax], 320 ± 3.7 ng/ml/hr; n = 7) and 2.5-fold (Vmax, 475 ± 5.7 ng/ml/hr; n = 7), respectively. The Kd of R3-36-16 for renin was similar when measured in the presence of hog (42.7 ± 2.9 pM; n = 7) or human TDP (42.4 ± 3.7 pM; n = 7). It made no significant difference whether purified human kidney renin or the MRC reference standard was used: 70 pM R3-36-16 incubated with 2.5 μM TDP produced 277 ± 13 (n = 12) and 284 ± 12 (n = 12) ng/ml/hr of angiotensin I (p > 0.05) in the presence of purified renin and MRC renin, respectively.

Moreover, the background production of angiotensin I from TDP using a similar concentration of inactive recombinant renin was not affected by R3-36-16 (control, 12.8 ± 0.96 vs 15.2 ± 1.1 ng/ml/hr; n = 12; p > 0.05).

Influence of R3-36-16 on the Kinetic Constants of Hog Tetradecapeptide Hydrolysis

At a near saturating concentration of R3-36-16 (0.7 nM), the Vmax doubled whereas the affinity of the substrate for renin (Km) remained unaffected (Table 1, Figure 2). Similar observations were made in the presence of lower concentrations of R3-36-16 (Figure 3; see Table 1). There was no significant difference in the kinetic constants when the Michaelis-Menten equation was applied to the data. This result was confirmed with the Allfit program constraining the data according to a noncompetitive model. Preincubation of the antibody with renin for 1 hour at 37°C did not change the kinetics of the reaction.

Influence of R3-47-10 on the Kinetic Constants of Hog Tetradecapeptide Hydrolysis

In the presence of hog TDP, the kinetics of the reaction at near saturating concentrations of R3-47-10 (0.7 and 7 nM) appeared complex. At low concentrations of substrate there was a clear inhibition of angiotensin I production. This inhibition was reversed into a stimulation by increasing the concentration of TDP (Figure 4). This antibody affected both Km and Vmax. Compared with the control, Km increased from 19.44

<table>
<thead>
<tr>
<th>Variable</th>
<th>Michaelis-Menten equation</th>
<th>Allfit analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (μM)</td>
<td>Vmax (ng/ml/hr)</td>
</tr>
<tr>
<td>Control</td>
<td>24.5 ± 4.2</td>
<td>1599 ± 70.2</td>
</tr>
<tr>
<td>R3-36-16 (7nM)</td>
<td>16.4 ± 2.2</td>
<td>2973 ± 98.1*</td>
</tr>
<tr>
<td>Control</td>
<td>25.9 ± 4.5</td>
<td>1658 ± 36.0</td>
</tr>
<tr>
<td>R3-36-16</td>
<td>17.6 ± 2.3</td>
<td>2219 ± 34.7*</td>
</tr>
<tr>
<td>35 pM</td>
<td>17.2 ± 2.3</td>
<td>2707 ± 43.4*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The Michaelis-Menten equation was applied using 32 values/curve. The means (9 points/curve) were used for the Allfit analysis.

*p < 0.01, as compared with the control value (by Dunnett's test).
± 0.66 to 97.30 ± 5.05 μM and $V_{\text{max}}$ also rose from 1012 ± 112.4 to 2178 ± 178.4 ng/ml/hr ($n = 8$). There was no significant difference between the two concentrations of the antibody. Preincubation of the antibody with renin for 1 hour at 37°C did not change the kinetics of the reaction.

**Effect of CGP 29287**

In contrast to the antibodies, CGP 29287, a peptidic transition-state analogue, proved to be a competitive inhibitor using the Michaelis-Menten equation or constraining the data with the Allfit program (Table 2, Figure 5). When hog TDP was used as a substrate, $V_{\text{max}}$ remained unchanged whereas $K_m$ increased threefold to sevenfold with 1 and 2.5 nM CGP 29287, respectively. The calculated $K_i$ was 0.34 nM.

**Cumulative Effect of CGP 29287 and R3-36-16**

CGP 29287 (1 and 2.5 nM) also inhibited renin activity, and to a similar degree, in the presence of 5 μM TDP and a wide range of antibody concentrations. This effect was found with both the partially inhibiting antibody R3-47-10 and with the stimulating antibody R3-36-16 (Figure 6).

A kinetic analysis was also performed with TDP as substrate and R3-36-16 and CGP 29287 added separately or combined (Figure 7). In the presence of 70 pM R3-36-16, $K_m$ did not change compared with the control whereas the velocity of the reaction increased by 160% ($p < 0.01$). CGP 29287 (1 nM) did not affect $V_{\text{max}}$, but it decreased the affinity by a factor of 5 ($p < 0.01$). In combination, R3-36-16 (70 pM) and CGP 29287 (1 nM) led to an increase in both $K_m$ and $V_{\text{max}}$, suggesting that the two compounds acted independently (Table 3). This conclusion was also supported when the data were constrained in the Allfit analysis.

**Effect of pH**

The stimulatory effect of 70 pM R3-36-16 was measured at different pH values corresponding to different degrees of ionization of substrate, enzyme, and antibody. The Michaelis-Menten constants of renin for TDP were lower at pH 5.7 and 9.0 than at pH 7.0, but the maximum velocity was not altered by the conditions of incubation (Table 4). In the presence of R3-36-16 and at pH 5.7, $K_m$ did not change, but $V_{\text{max}}$ increased in the same proportion (+180%) as at pH 7.0. In
TABLE 2. Effect of CGP 29287 on the Kinetic Constants of the Enzymatic Reaction

<table>
<thead>
<tr>
<th>Variable</th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (ng/ml/hr)</th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (ng/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.1 ± 2.7</td>
<td>1424 ± 45.4</td>
<td>23.9 ± 3.1</td>
<td>1484 ± 53.2</td>
</tr>
<tr>
<td>CGP 29287</td>
<td>1 nM</td>
<td>70.6 ± 7.9*</td>
<td>1420 ± 52.4</td>
<td>79.8 ± 9.5*</td>
</tr>
<tr>
<td></td>
<td>2.5 nM</td>
<td>164.4 ± 13.8*</td>
<td>1435 ± 49.7</td>
<td>179.0 ± 20.1*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The Michaelis-Menten equation was applied using 18 points/curve. The means (9 points/curve) were used for the Allfit analysis.

*p < 0.01, as compared with the control value (by Dunnett’s test).

Discussion

Our data confirm that the monoclonal antibodies R3-47-10 and R3-36-16 are potent inhibitors of the enzymatic reaction of renin with the natural substrate, angiotensinogen. Both antibodies inhibit renin activity completely at concentrations above 700 pM. However, their effects are clearly differentiated when a small synthetic substrate, TDP, is used. R3-47-10 at low substrate concentrations inhibited angiotensin I production from hog TDP (Table 7).

Specificity of the Stimulatory Effect of R3-36-16

The effects of R3-36-16 were compared with that of monoclonal antibodies against phosphatidylcholine or eglin purified by the same procedure. None of these antibodies at any of the concentrations tested produced any effect on angiotensin I production from hog TDP (Table 7).
production from hog or human TDP, whereas under the same experimental conditions R3-36-16 augmented angiotensin I formation. It is unlikely that R3-36-16 or R3-47-10 interacts directly with the catalytic site inside the enzymatic cleft for the following reasons: 1) Both bind equally well to active and inactive renin; 2) the stimulating effect of these antibodies is distinct from and additional to the action of CGP 29287, an inhibitor competing with the substrate for the active site of human renin (see Figure 7).

An explanation for the increase in renin activity induced by R3-36-16 with TDP as substrate might be that this antibody binds to and activates an inactive form of renin present in minute amounts in the renin preparation used in these experiments. This explanation appears unlikely since R3-36-16 did not activate preparations of inactive renin from human chorionic cells or recombinant DNA. Moreover, contamination of the R3-36-16 preparation with a reninlike activity can be excluded, as there was no significant generation of angiotensin I when R3-36-16 and TDP were incubated for 1 hour in the absence of renin. Furthermore, R3-47-10 and other monoclonal antibodies directed against renin or other proteins (eglin, phosphatidylcholine), produced and purified by the same technique as for R3-36-16, did not stimulate angiotensin I, suggesting that the effect of R3-36-16 was not due to contamination with a proteinase that activates renin.

An alternative explanation for the stimulation induced by R3-36-16 may be that this antibody affects the structural conformation of the whole protein. The mechanism of entry of the N-terminal part of angiotensinogen into the cleft is thought to require retroflexion of the flap overlying the active site (Positions 79–91). Since R3-36-16 is a very potent inhibitor of renin activity, it would be expected to bind close to the active site. For example, it may increase the access and turn-
TABLE 5. Effect of the Combination of the Two Antibodies on Angiotensin I Production

<table>
<thead>
<tr>
<th>Variable</th>
<th>Angiotensin I (ng/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>520 ± 9.50</td>
</tr>
<tr>
<td>R3-36-16 (7 nM)</td>
<td>1108 ± 31.0*</td>
</tr>
<tr>
<td>R3-36-16 (7 nM) + R3-47-10 (0.7 nM)</td>
<td>1124 ± 34.3*</td>
</tr>
<tr>
<td>R3-36-16 (7 nM) + R3-47-10 (7 nM)</td>
<td>1097 ± 48.3*</td>
</tr>
<tr>
<td>R3-36-16 (7 nM) + R3-47-10 (70 nM)</td>
<td>927 ± 26.1*</td>
</tr>
<tr>
<td>R3-36-16 (7 nM) + R3-47-10 (700 nM)</td>
<td>1037 ± 28.4*</td>
</tr>
<tr>
<td>R3-47-10 (7 nM)</td>
<td>390 ± 5.3*</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 8). The concentration of hog tetradecapeptide was 15 µM. *p < 0.01, as compared with the control value (by Dunnett’s test).

TABLE 6. Effect of Sequential Combination of the Antibodies

<table>
<thead>
<tr>
<th>Tetradecapeptide (µM)</th>
<th>1st incubation (antibody in nM)</th>
<th>2nd incubation (antibody in nM)</th>
<th>Angiotensin produced (ng/ml/1 hr)</th>
<th>Angiotensin produced (ng/ml/2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>342 ± 13</td>
<td>574 ± 15</td>
</tr>
<tr>
<td>5</td>
<td>R3-36-16 (7)</td>
<td>R3-47-10 (7)</td>
<td>698 ± 15</td>
<td>1025 ± 13</td>
</tr>
<tr>
<td>5</td>
<td>R3-47-10 (7)</td>
<td>R3-36-16 (7)</td>
<td>209 ± 10</td>
<td>998 ± 21</td>
</tr>
<tr>
<td>15</td>
<td>—</td>
<td>—</td>
<td>752 ± 16</td>
<td>1300 ± 68</td>
</tr>
<tr>
<td>15</td>
<td>R3-36-16 (7)</td>
<td>R3-47-10 (7)</td>
<td>1417 ± 36</td>
<td>2656 ± 60</td>
</tr>
<tr>
<td>15</td>
<td>R3-47-10 (7)</td>
<td>R3-36-16 (7)</td>
<td>549 ± 12</td>
<td>921 ± 26</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 12). The second antibody was added 1 hour after the first (concentration is shown in parentheses). Angiotensin I was measured after a 1- or 2-hour incubation. *p < 0.05, as compared with the effect of R3-47-10 alone at 2 hours (by Dunnett’s test).
antibodies may affect renin differently, despite the very close proximity of their respective binding sites, their similar binding affinities, and their similar inhibition of renin activity when the natural substrate is used. Since the formation of angiotensin I from TDP is actually augmented by R3-36-16 and to some degree by R3-36-10, these antibodies may induce a conformational change in renin. This structural modification may allow an increased access of TDP to the catalytic site or a more rapid release of the product from the enzymatic cleft, or both effects. The effects of these antibodies on catalytic activity are good examples of the complexity of the interaction between monoclonal antibodies and enzymes.

Acknowledgments

We thank Joseph Rahuel for the isolation and purification of human renin and angiotensinogen, Lilian Hartman and Steven E. Whitebread for valuable assistance, Dr. Frédéric Cumin for his critical discussion of the results, Myriam M. de Gasparo for making the graphs, and Jeanine Bollecker for typing the manuscript.

References

18. Cheng YC, Prusoff WH. Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099-3108
Mechanism of inhibition of human renin by monoclonal antibodies.
M M de Gasparo, J M Wood and C H Heusser

Hypertension. 1988;11:209-216
doi: 10.1161/01.HYP.11.3.209

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on
the World Wide Web at:
http://hyper.ahajournals.org/content/11/3/209