Comparative Effects of Three Different Potent Renin Inhibitors in Primates

Jean-Paul Clozel, Walter Fischli

The goal of the present study was to compare the effects of three potent reference renin inhibitors (remikiren, CGP 38560A, and enalkiren) in sodium-depleted normotensive squirrel monkeys. In these monkeys, arterial pressure was measured in the conscious state with a telemetry system. Oral and intravenous maximal effective doses of the three renin inhibitors were compared in parallel groups of monkeys. In additional experiments, remikiren was given on top of either CGP 38560A or enalkiren in the same animals. Finally, the three drugs were compared with the angiotensin converting enzyme inhibitor cilazapril. The effects of the three drugs on the plasma components of the renin-angiotensin system (plasma renin activity, immunoreactive renin, and immunoreactive angiotensin II concentrations) were also measured. Our results show that remikiren was as effective as cilazapril and markedly more effective than CGP 38560A or enalkiren in reducing arterial pressure in our monkey model. Interestingly, these differences in arterial pressure could not be explained by differences of in vitro potency or different biochemical changes of the plasma components of the renin-angiotensin system, because the inhibitors all reduced immunoreactive angiotensin II to similarly low levels. One possible explanation is that, in our model, remikiren in contrast to CGP 38560A and enalkiren is able to inhibit renin in a functionally important extraplasmatic compartment.

KEY WORDS • kininase II • primates • angiotensin II • cilazapril • hypertension, sodium-dependent • renin

Renin inhibitors (for review, see References 1 through 5) are presently proposed as new antihypertensive drugs. Like angiotensin converting enzyme (ACE) inhibitors, they can reduce arterial pressure by blocking the renin-angiotensin system. However, these drugs should theoretically be devoid of the side effects of ACE inhibitors such as cough or angioneurotic edema. These side effects have been attributed to the potentiation of bradykinin effects, secondary to the inhibition of ACE, which cleaves not only angiotensin I (Ang I) but also bradykinin.

CGP 38560A, enalkiren, and remikiren have been described as potent renin inhibitors in primates. However, clinical results in hypertensive patients are controversial. Only remikiren has been shown to lower arterial pressure in hypertensive patients under a normal-sodium diet after oral application. In hypertensive patients, CGP 38560A had a limited antihypertensive effect compared with captopril when given intravenously. Enalkiren was shown to have an antihypertensive effect after high intravenous doses or after long-term intravenous infusion. No oral testing was performed.

The goal of the present study was to perform a comparison of these three drugs in primates. For this purpose, the effects of the three drugs on arterial pressure and on the different plasma components of the renin-angiotensin system were evaluated in conscious, sodium-depleted squirrel monkeys.

Methods

In Vitro Experiments

The in vitro potency of the compounds was evaluated in EDTA plasma of humans and squirrel monkeys under the same conditions as the plasma renin activity (PRA) was measured (trapping assay, see below) and against purified human renal renin. For the plasma assay, the inhibitors were made up as a solution of 85 μM in dimethyl sulfoxide and diluted further by the antibody solution 10- to 100 000-fold (see below).

The plasma assay for measuring the in vitro potencies of the renin inhibitors was performed at pH 7.4 and was similar to the trapping assay used for the estimation of PRA developed by Nussberger et al. The assay consisted of (1) 75 μL human or squirrel monkey EDTA-plasma; (2) 75 μL trapping buffer (0.2 M Tris-HCl, pH 7.5, including 0.3% human serum albumin) with or without Ang I standards; (3) 7 μL of 3 M Tris-HCl, pH 7.2, including 200 mM EDTA; and (4) 3 μL antisera (AS MAUROY, 1:500) in trapping buffer. To save pipetting, we premixed solutions 3 and 4 to give the antibody solution, which was also used for the dilution of the 85-μM inhibitor stock solution.

The antibody used was a fast-binding polyclonal Ang I antibody raised at Roche (Ang I AS MAUROY) with an IC50 of 60 pg per assay tube or 290 pM, which is capable of trapping and protecting the formed Ang I in the absence of potentially disturbing angiotensinase inhibitors.
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July 1993

Hemodynamic Experiments

The enzyme assay was started by incubation of mixtures (1), (2), and (4) for PRA samples and (2), (3), and (4) for the Ang I standard curve at 37°C. It was stopped 2 hours later by addition of the remaining solution (1) or (2) and 1 mL ice-cold barbitone acetate (0.06 M, pH 8.6), including 0.3% human serum albumin containing 5000 cpm 125I-Ang I, a solution that diluted the Ang I antibody body to the correct radioimmunoassay concentration. After an incubation of 24 hours at 4°C, the radioimmunoassay was terminated by addition of 300 µL water containing 2.5% charcoal and 0.25% dextran T70 and spinning at 3000g for 20 minutes. The supernatant was counted in a gamma counter. Maximum enzymatic Ang I production (which is identical to regular PRA) was measured in the absence of inhibitors and minimum Ang I production (maximal inhibition) in the presence of 10 µM remikiren. PRA for both human and squirrel monkey plasma was approximately 2 ng Ang I/mL per hour. The percentage of inhibition was calculated at each concentration point, and the concentration of renin inhibitor that inhibited enzymatic activity by 50% (IC50) was determined.

For use in the in vitro assay system, renin was purified in one step from infarcted human kidneys by immunofinity chromatography as described.21 The material so prepared was used directly for in vitro assays and had a specific activity of 200 Goldblatt units (GU) per milligram of protein.

The buffer used for the in vitro assay was 0.1 M sodium phosphate, pH 7.4, containing 0.1% bovine serum albumin and 1 mM disodium-EDTA. The incubation mixture consisted of (1) 100 µL renin in assay buffer (0.5 mM/GU/mL), (2) 30 µL human tetradecapeptide renin substrate in 10 mM HCl (10 µM), (3) 10 µL hydroxyquinoline sulfate in water (30 mM), (4) 145 µL assay buffer, and (5) 15 µL renin inhibitor in dimethyl sulfoxide at various concentrations. The samples were incubated at 37°C for 3 hours. The generated Ang I was estimated by a commercially available radioimmunoassay kit (Baxter Healthcare Corp, Cambridge, Mass).

Measurement of immunoreactive angiotensin II. Plasma ir-Ang II was quantified after extraction of plasma on Sep-Pak C18 cartridges as described.14 The polyclonal antibody used for the measurement of ir-Ang II (Ang II AS No. 923) was raised at F. Hoffmann-La Roche Ltd, Basel, Switzerland. The IC50 value was 5.5±0.31 fmol per assay tube (n=8), and the cross-reactivities against other angiotensin peptides were Ang II, 100%; Ang I, 0.37±0.10%; Ang I (2-10), <0.02%; and Ang III, <0.02%. The antibody used for the measurement of ir-Ang II specifically recognizes an epitope of mature renin and does not cross-react with inactive prorenin but does cross-react fully with active renin even when inhibited.

Measurement of immunoreactive active renin. Ir-AR was measured in separate groups of animals, according to the method of Ménard et al,24 with an immunoradiometric assay commercially available at Diagnostics Pasteur, Marnes-La-Coquette, France. The monoclonal antibody used to detect active renin specifically recognizes an epitope of mature renin and does not cross-react with inactive prorenin but does cross-react fully with active renin even when inhibited.

Study Design

Comparison of the three drugs (parallel groups). The three drugs were compared in parallel groups of monkeys that received either 10 mg/kg po or 1 mg/kg iv as a solution (dissolved in water). These doses were the
maximal effective doses as shown by the absence of additional effects when higher doses were given.

Because the experiments in parallel groups showed clear differences among the three renin inhibitors on arterial pressure, we performed additional experiments. First, either CGP 38560A or enalkiren (10 mg/kg po or 1 mg/kg iv) was administered. Two hours later, when the plateau effect of these drugs on arterial pressure was reached, a second dose of either the same compound or of remikiren was given, and arterial pressure was monitored for 2 more hours. Finally, because the three drugs had a different in vitro potency to inhibit renin, we compared the effects of the three drugs given intravenously at doses inversely proportional to their in vitro potency: 0.1, 0.25, and 1 mg/kg for remikiren, CGP 38560A, and enalkiren, respectively.

Combination with cilazapril. To compare the effects of the three renin inhibitors with those of cilazapril, we gave 10 mg/kg po of the three renin inhibitors, and after 2 hours, we added 10 mg/kg po of cilazapril, a long-acting ACE inhibitor.25 Thereafter, arterial pressure was recorded for 2 more hours.

Ex Vivo Measurements

PRA and ir-AR were measured in separate groups of sodium-depleted squirrel monkeys at baseline (before drug administration) and 30, 60, 120, and 240 minutes after oral administration of placebo or 10 mg/kg remikiren, CGP 38560A, or enalkiren. In addition, in other groups of sodium-depleted monkeys, plasma ir-Ang II was measured 30 minutes after administration of placebo or of the three renin inhibitors (10 mg/kg po).

Statistical Analysis

All data are given as mean±SEM. Effects of the drugs were compared using a Newman-Keuls Test. To avoid bias due to multiple comparisons, we performed an analysis only at the time of peak effect (60 minutes). Because the peak effect for biochemical changes occurs between 30 and 60 minutes, we performed a statistical analysis at these two time points. An analysis was also performed at 240 minutes but should only be considered as descriptive. A value of P<.05 was considered significant.

Results

In Vitro Potency

In vitro, the three renin inhibitors were all potent inhibitors of purified human renin, but CGP 38560A and remikiren were clearly superior in this system compared with enalkiren (Table). However, when the potency was measured in human plasma under close to physiological conditions, remikiren, enalkiren, and CGP 38560A were 7-, 20-, and 35-fold less potent, respectively. In squirrel monkey plasma, all the compounds were 5- to 10-fold less potent than in human plasma.

In Vivo Effects on Arterial Pressure

There were marked differences of efficacy among the three renin inhibitors after oral (Fig 1) and intravenous

### In Vitro Potency of the Three Renin Inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_50$ for enzymatic inhibitory activity (nM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Human recombinant renin (pH 7.4)</td>
</tr>
<tr>
<td>Remikiren (Ro 42-5892)</td>
<td>0.41±0.05 (n=15)</td>
</tr>
<tr>
<td>CGP 38560A</td>
<td>0.18±0.02 (n=4)</td>
</tr>
<tr>
<td>Enalkiren (A 64662)</td>
<td>2.5±0.6 (n=5)</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
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Control, H2O iv (n=9) CGP38560A, 1mg/kg iv (n=16)
Remikiren, 1mg/kg iv (n=8) Enalkiren, 1 mg/kg iv (n=5)

FIG 2. Line graph shows effects of placebo and three renin inhibitors given intravenously on mean arterial pressure (MAP) in conscious, sodium-depleted squirrel monkeys. Mean±SEM of differences from baseline are represented. Statistical analysis was performed at 60 minutes. ***P<.001, **P<.01 vs placebo. Baseline MAP values were 96±2, 96±3, 96±2, and 91±2 mm Hg in control, enalkiren, CGP38560A, and remikiren groups, respectively.

(Fig 2) administration. With 10 mg/kg po, remikiren, CGP 38560A, and enalkiren decreased arterial pressure by 35±1, 14±2, and 11±2 mm Hg, respectively. Similarly, after 1 mg/kg iv, the three drugs decreased arterial pressure by 33±3, 17±3, and 10±2 mm Hg, respectively.

The better efficacy of remikiren was confirmed by oral (Fig 3) and intravenous (Fig 4) experiments, during which remikiren was given on top of CGP 38560A or enalkiren in the same animals. Remikiren reduced significantly arterial pressure further than a second dose of the other two inhibitors.

When equipotent doses (calculated from in vitro experiments) of 1, 0.25, and 0.1 mg/kg were administered intravenously, similar differences were seen as described above (Fig 5).

The arterial pressure after remikiren was not further decreased by the addition of cilazapril (Fig 6). In contrast, there was additional lowering of blood pressure when cilazapril was added to CGP 38560A and enalkiren.

Ex Vivo Measurements

Despite different in vitro potencies, the three renin inhibitors decreased PRA to a similar extent after a dose of 10 mg/kg po (Fig 7). This decrease, maximal at 30 minutes, was associated with a reactive rise of ir-AR, which was similar with remikiren and CGP 38560A and smaller with enalkiren. PRA returned progressively to baseline within 2 hours, with enalkiren overshooting at 240 minutes. In contrast, ir-AR was still increased at 240 minutes with the three renin inhibitors. In addition, the three renin inhibitors decreased plasma ir-Ang II significantly to low levels with this dose at the time of the biggest PRA inhibition (Fig 8).

Discussion

The present study shows that the three renin inhibitors remikiren, CGP 38560A, and enalkiren have very different efficacies for lowering arterial blood pressure in conscious, sodium-depleted squirrel monkeys. Remikiren has a clearly superior efficacy to lower blood pressure than both CGP 38560A and enalkiren. This is
very surprising, because one could have expected similar efficacy with drugs having a common mechanism of action, eg, renin inhibition. In addition, the three drugs previously have been shown to be potent renin inhibitors and to lower arterial blood pressure in sodium-depleted primates. However, the in vitro potency of these compounds is very much influenced by the methodology used. This is not completely unexpected, because the measured potency is an apparent affinity influenced greatly by the protein binding, which reduces the concentration of the available free drug. It is worth noticing that addition of nonphysiological organic compounds, such as the angiotensinase inhibitors hydroxyquinoline sulfate or phenylmethylsulfonyl fluoride, which are frequently added to plasma assays, is able to reverse the protein binding of the renin inhibitors. This results in an increase of the apparent affinity, which no longer reflects the physiological potency. The same argument holds true for measuring ex vivo PRA after treatment with a renin inhibitor, which may result in similar artifacts of the measured renin inhibition (see References 26 and 27). Hence, for the correlation of in vitro potency with in vivo (blood pressure) and ex vivo (PRA) effects, it is important (1) to estimate the apparent potency of the renin inhibitors in vitro in plasma and ex vivo on PRA using the same methodology and (2) to choose a methodology that is as close as possible to physiological conditions. Our methodologies are compatible and physiological because we omitted angiotensinase inhibitors but protected the formed Ang I by trapping it with Ang I antibodies. This so-called trapping methodology is described as being devoid of major artifacts (see also, References 26 and 27), therefore measuring the apparent potency of the compounds in vitro and ex vivo (PRA) under conditions that should allow an extrapolation to in vivo activity. Under these conditions, the observed potency in human plasma is 3.5-fold and 14-fold less than that described originally for enalkiren ($IC_{50}$, 14 nM) and CGP 38560A ($IC_{50}$, 0.7 nM), respectively.
FIG 5. Line graph shows comparative effects on mean arterial pressure (MAP) of equipotent doses of remikiren, CGP38560A, and enalkiren. Doses were calculated according to the in vitro potency of the drugs.

It is interesting to note that, with the use of our plasma methodology, the in vitro potencies are 7-, 20-, and 35-fold less for remikiren, enalkiren, and CGP 38560A when compared with an in vitro assay using purified renin (Table). This would indicate that CGP 38560A displays the strongest plasma protein binding of the three inhibitors and remikiren the least, which results in a change of the potency rank order and suggests that remikiren should be the most potent of the three inhibitors in human trials. The same potency rank order is found in squirrel monkey plasma but with 5- to 10-fold lower potencies of the three renin inhibitors (Table).

In sodium-depleted squirrel monkeys, after oral administration of the three inhibitors using the same high dose of 10 mg/kg, remikiren clearly reduced arterial blood pressure more than enalkiren and CGP 38560A (Fig 1). It is worth mentioning that the oral dose used represents the maximum effective oral dose of remikiren in this model, as shown before.14 This is also true for enalkiren and CGP 38560A, because a second administration given on top of the first dose did not

FIG 6. Line graph shows effects on mean arterial pressure (MAP) of cilazapril given on top of CGP38560A, enalkiren, or remikiren. Groups of four animals were used. The three renin inhibitors were given first (1 mg/kg iv); after 2 hours, cilazapril was added. Cilazapril had a marked additive effect after CGP38560A or enalkiren but not after remikiren. ***P<.01 vs CGP38560A or enalkiren.
reduce arterial pressure further. To eliminate the possibility that the observed differences were due to interindividual differences, we performed experiments during which remikiren was given on top of a treatment with either enalakiren or CGP 38560A (Figs 3 and 4). These experiments confirmed that, in the same animals, enalakiren and CGP 38560A were not able to reduce arterial pressure to the same extent as remikiren.

However, all three compounds were able to affect the different plasmatic components of the renin-angiotensin system after the same oral dose. The effects on PRA were similar with remikiren and CGP 38560A and less with enalakiren (Fig 7). Similarly, the increase of ir-AR was more marked with the former compounds and less with the latter (Fig 7). This increase of ir-AR is thought to be due to the interruption of the negative feedback loop by reduction of Ang II. Indeed, plasma ir-Ang II was found to be reduced at the time of the biggest PRA decrease compared with the control levels (Fig 8). Thus, there seems to be a logical sequence of events, such as PRA inhibition resulting in reduction of Ang II leading to an increased release of renin. Similar biochemical effects have been described previously in experimental models and in humans. But interestingly, these changes in plasma biochemical variables, especially ir-Ang II, do not reflect the observed blood pressure changes in our model.

Nonparallelisms between biochemical changes of the renin-angiotensin system and changes of blood pressure by ACE and renin inhibitors have been observed before (for review, see Reference 4). One of the most prominent cases was described after long-term treatment of spontaneously hypertensive rats with enalapril, which led to chronically lowered blood pressure but even overshooting plasmatic Ang II levels. Indeed, after short-term treatment with remikiren, we also observe long and profound blood pressure decreases but only transient changes in biochemical variables as described before. Similar results were obtained in hypertensive patients.

To eliminate the differences in oral absorption, the three renin inhibitors were also tested after intravenous administration. After the same high dose of 1 mg/kg, a distinct difference was still observed among the inhibitors in their ability to reduce arterial blood pressure (Fig 2). Again, when tested subsequently in the same animals, remikiren reduced blood pressure significantly more than the other two inhibitors (Fig 4). Moreover, when the three inhibitors were given with doses that should result in similar effects according to the in vitro potencies, blood pressure responses were very different (Fig 5). Whereas remikiren reduced arterial pressure by 20 mm Hg at this dose, the efficiency of the other two inhibitors was much less.

The differences of blood pressure-lowering effects are unlikely to be due to technical problems. We have
used conscious animals to avoid effects of anesthesia. Moreover, we used a telemetry system to avoid effects of stress. Arterial pressure could be measured without any human presence close to the monkeys. This is the reason why it is difficult to compare the absolute changes of arterial pressure obtained in our study to those obtained in studies in which the drugs were tested during anesthesia or with animals restrained. In these conditions, both CGP 38560A and enalapril were more effective than captopril.

Remikiren decreased arterial pressure to the same level as cilazapril, a new long-acting ACE inhibitor. These results have been previously described. In contrast, both CGP 38560A and enalapril were less effective than cilazapril in our model. A similar observation has been made for CGP 38560A. However, in other studies, both CGP 38560A or other renin inhibitors were shown to be as effective as ACE inhibitors. Interestingly, in hypertensive patients, CGP 38560A was less effective than captopril. Results in patients with enalapril are controversial. The reason for these discrepant findings is unclear but could be related to differences in species, methodology, or both.

Differences of efficacy have not been reported for ACE inhibitors. However, ACE is an endothelial enzyme that is perhaps easier to reach than tissular renin (because to inhibit renin in plasma is not sufficient to reduce arterial pressure). In monkeys, remikiren has a distribution volume approximately 10 times the blood volume (unpublished observation), which is larger than the distribution volume of CGP 38560A or enalapril, pointing to an extensive tissular distribution of remikiren compared with both of the other renin inhibitors. These differences of tissular distribution could explain the different antihypertensive efficacy of the three drugs despite similar plasmatic biochemical changes. This would assume that renin inhibition needs to occur at the tissular level for arterial pressure to decrease, at least in our model.

In humans, a major part of Ang I production has been shown to be tissular. In addition, it is possible to measure Ang I formation in vivo using isolated organs and in tissue culture. Interestingly, in vivo Ang I formation by arterial vessels can be blocked by renin inhibitors, which also shows that renin is the main enzyme responsible for the Ang I production. We could show that remikiren is present in various tissues, including the vessel wall, where it has a very long half-life (unpublished data). Therefore, it is tempting to believe that remikiren is acting by inhibiting renin present in an extraplasmatic compartment.

The fact that renin inhibitors could have different antihypertensive efficacy underlines the necessity to interpret with caution future results obtained in hypertensive patients treated with renin inhibitors, because it may not be possible to extrapolate from one renin inhibitor to another. Moreover, clinical trials including ACE and renin inhibitors need to be performed to compare the therapeutic benefit of both approaches.

Acknowledgments

We thank Evelyn Dormbier, Josiane Rein, Patrick Hess, Astrid Zimmermann, and Paul Mosy for excellent technical help; Drs Wolfgang Wöstl, Heinz Stadler, and Eric Vieira for the synthesis of the three renin inhibitors; and Alexandra Zürer for secretarial assistance.

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Comparative effects of three different potent renin inhibitors in primates.
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Hypertension. 1993;22:9-17
doi: 10.1161/01.HYP.22.1.9

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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