Ciprokiren (Ro 44-9375)
A Renin Inhibitor With Increasing Effects on Chronic Treatment

Walter Fischli, Jean-Paul Clozel, Volker Breu, Stefan Buchmann, Salima Mathews, Heinz Stadler, Eric Vieira, Wolfgang Wostl

Abstract The present study characterizes the new transition-state renin inhibitor ciprokiren (Ro 44-9375) in squirrel monkeys. Arterial blood pressure was monitored by telemetry in freely moving, chronically instrumented conscious animals. In vitro at pH 7.4, ciprokiren inhibited human renin in buffer and human plasma with an IC₅₀ of 0.07 and 0.65 nmol/L, respectively. It was equipotent against primate plasma renin and also inhibited plasma renin from dog and guinea pig in the nanomolar range (IC₅₀, 29 and 65 nmol/L, respectively). After acute oral administration it reduced arterial blood pressure dose dependently in normotensive sodium-depleted and cyclosporin-induced hypertensive squirrel monkeys, starting with the minimal oral dose of 3 µg/kg. Daily oral doses of 1 µg/kg showed a progressive blood pressure decrease, with a maximal response reached after 1 week. The drug could also be applied transdermally with similar hemodynamic effects without any decrease of plasma renin activity or plasma immunoreactive angiotensin II. Thus, ciprokiren is characterized in squirrel monkeys as a renin inhibitor with high in vivo potency that might act mainly in the tissular compartment. (Hypertension. 1994;24:163-169.)

Key Words • renin • primates • angiotensin II • angiotensin converting enzyme • renin-angiotensin system

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure and fluid homeostasis. It generates its effector hormone angiotensin II (Ang II) in two steps. In a first step, renin cleaves its protein substrate angiotensinogen to release the biologically inactive decapetide Ang I. In a second step, Ang I is further cleaved to the octapeptide Ang II by angiotensin-converting enzyme (ACE). Ang II finally interacts with at least two different receptor subtypes, AT₁ and AT₂. Whereas AT₁ seems to be responsible for most of the known functions of Ang II, the function of AT₂ is unclear at present.¹

ACE inhibition represents a major advance in the treatment of hypertension and congestive heart failure.²³ Today, ACE inhibitors are also the drugs of choice after myocardial infarction because they prolong life expectancy.₄⁵ However, despite this success, their use is associated with some side effects, such as cough and angioedematous edema. This is most probably because of bradykinin accumulation,⁶ since ACE is also involved in bradykinin degradation.⁷ Furthermore, ACE can be bypassed by other enzymes such as chymase⁸ or chymostatin-sensitive Ang II–generating enzyme (CAGE).⁹ which may reduce the efficacy of ACE inhibitors.¹⁰

Alternatively, the RAS may be blocked more specifically using Ang II receptor blockers or renin inhibitors. At present, several AT₁ receptor blockers are under clinical investigation. The development of renin inhibitors has been compromised by low oral bioavailability because of high liver first-pass and extensive biliary excretion.¹² One exception seems to be the inhibitor A 72,517, which shows high bioavailability in a variety of species¹² and is presently under clinical investigation.¹³ Overall, AT₁ blockers and renin inhibitors are able to reduce high blood pressure in hypertensive patients to a similar extent as ACE inhibitors.¹⁴⁻¹⁶ However, in congestive heart failure losartan as a prototypical AT₁ blocker showed only limited effects on pulmonary wedge pressure,¹⁷ whereas the renin inhibitors enalkiren¹⁸ and remikiren¹⁹ were very effective.

Previously, we have described remikiren (Ro 42-5892) as a potent orally active renin inhibitor in primates.²⁰⁻²² In squirrel monkeys it was as effective as ACE inhibitors or neutralizing renin antibodies in a sodium-depleted state but reduced high blood pressure significantly more than the other two pharmacological interventions in a cyclosporin-induced hypertensive state.²³ Interestingly, other renin inhibitors such as enalkiren and CGP 38560A were not as effective as remikiren.²⁴ In hypertensive patients clinical studies have shown that remikiren given intravenously is as effective in lowering blood pressure as an established oral dose of captopril.²⁵

In the present study we characterized the new renin inhibitor ciprokiren (Ro 44-9375) in vitro and in vivo and compared it with remikiren. The results pointed toward a long-lasting tissular effect of ciprokiren, so we evaluated the effects of long-term low doses. Hence, we could show that after long-term oral administration of very low doses or transdermal application, ciprokiren was able to lower blood pressure as much as after high oral doses given acutely.
Fig 1. Diagram shows chemical structure of ciprokiren (Ro 44-9375).

Methods

Drugs
Ciprokiren is a transition-state renin inhibitor related to remikiren with a molecular weight of 729 and the structure (S)-2-benzyl-N-[(S)-1-{(1S,2R,3S)-1-cyclohexylmethyl-3-cyclopropyl-2,3-dihydropyrrol-2-carboxamido}]-2-(imidazol-4-yl)-ethyl]-3-[1-methyl-1-(morpholin-4-ylcarbonyl)-ethylsulfonamido]-propionamide (Fig 1). The compound is a derivative of a hydroxy metabolite that was formed from remikiren by human liver microsomes. The solubility of the methanesulfonate is greater than 200 mg/mL at pH 3 and 2.8 mg/mL at pH 7.4. The compound is stable in human plasma, human gastric juice, and human intestinal juice at 37°C for 24 hours.

In Vitro Experiments
We evaluated the in vitro renin inhibitory potency of ciprokiren at pH 7.4 in plasma of various species and against recombinant human renin. We estimated selectivity by measuring inhibition of the closely related enzymes porcine pepsin and bovine cathepsin D at pH 2.8.

We measured the inhibition of plasma renin activity (PRA) in vitro using the trapping assay as used for the estimation of PRA ex vivo (see below). The methodology has been previously described and allows the evaluation of the affinity that comes closest to in vivo conditions. It avoids the use of angiotensinase inhibitors but protects the in vitro-generated Ang I by trapping with Ang I antibodies. Angiotensinase inhibitors may create an artificial "in vitro potency" that does not reflect in vivo conditions.

For use in the in vitro assay, recombinant human renin was cloned and expressed at F. Hoffmann-La Roche Ltd. It showed intrinsic activity similar to that of purified human renal renin when the synthetic tetradecapeptide was used as renin substrate. The procedures for the in vitro renin assay and the cathepsin D and pepsin assays have been published.

Hemodynamic Experiments
All experimental procedures followed were in accordance with institutional guidelines. The effects of ciprokiren on arterial pressure and heart rate were measured with a telemetry system in conscious squirrel monkeys of either sex weighing 400 to 700 g. The animals were either sodium depleted by diet or rendered hypertensive by chronic (more than 3 weeks) treatment with cyclosporin A as described previously.

Arterial pressure was monitored in nonrestrained conscious squirrel monkeys (without telemetry systems). Plasma measurements were performed after a single oral administration of 10 mg/kg ciprokiren. Blood was sampled at different time points from the same animals for the evaluation of the time course of the variables. However, because of the limited volume that could be taken from these rather small monkeys, only one type of variable could be measured in each monkey group. Blood samples were taken by direct puncture of the femoral vein for either PRA, immunoreactive renin, or immunoreactive Ang II measurements. For PRA and immunoreactive renin, the blood was anticoagulated with EDTA (10 mmol/L final concentration). For immunoreactive Ang II, a cocktail of ciprokiren and cilazapril (1 μmol/L final concentration) was added to prevent in vitro generation of Ang II. The blood was then centrifuged at 3000g for 10 minutes at 4°C to separate the plasma, which was finally frozen at −20°C until use.

Measurement of PRA
As for in vitro experiments, we used the trapping methodology of Poulsen and Joergensen. The method measures PRA by a radioimmunologic microassay based on Ang I trapping by antibodies. The method was adapted and described in detail by Nussberger et al. A fast-binding Ang I antibody was used that was raised in rabbits at F. Hoffmann-La Roche Ltd (Ang I-AS MAUROY) and displayed an IC50 of 50 pg per assay tube, which is ideal for the measurement of PRA in the range of 100 to 30 000 pg Ang I/mL per hour.

Measurement of Immunoreactive Renin
Immunoreactive renin was measured by the method of Ménard et al with a commercially available immunoradiometric assay (Diagnostics Pasteur). The assay measures specifically mature renin and does not cross-react with inactive prorenin but cross-reacts fully with mature renin inhibited by our renin inhibitors.

Measurement of Immunoreactive Ang II
Before measurement, Ang II was extracted from plasma by Sep-Pak C18 cartridges as described. The polyclonal antibody used for the radioimmunoassay (Ang II-AS 923) was raised in rabbits at F. Hoffmann-La Roche Ltd. It was very sensitive, with an IC50 value of 5.5±0.31 fmol per assay tube (n=8), and extremely specific for Ang II, with essentially no cross-reactivities against other angiotensin peptides (eg, Ang I, Ang III; see Reference 20).

Statistical Analysis
All data are expressed as mean±SEM. We assessed the effect on blood pressure by ANOVA, taking into account the factors dose and time compared with the placebo group. We
Inhibitory Potency and Selectivity of Ciprokiren

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>Assay, pH</th>
<th>IC_{50}, nmol/L</th>
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<tr>
<td>recRenin</td>
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<td>0.07</td>
</tr>
<tr>
<td>Plasma renin</td>
<td>Human</td>
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<td>0.65</td>
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<tr>
<td>Squirrel monkey</td>
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<tr>
<td>Guinea pig</td>
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</tr>
<tr>
<td>Dog</td>
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<tr>
<td>Rat</td>
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<td>Pepsin</td>
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<tr>
<td>Cathepsin D</td>
<td>Bovine</td>
<td>2.8</td>
<td>16 000</td>
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assessed the biochemical effects of ciprokiren by comparing the variables in the treated groups with the control groups by an unpaired t test.

Results

Ciprokiren is a very potent and specific renin inhibitor in vitro (Table). It inhibited recombinant human renin at pH 7.4 in the absence of plasma with an IC_{50} of 0.07 nmol/L, and discriminated the closely related enzymes pepsin and cathepsin D from human renin by a factor of more than 150 000 (IC_{50}, >100 and 16 μmol/L, respectively). In the presence of plasma, the compound inhibited human renin at pH 7.4 with an IC_{50} of 0.65 nmol/L and was roughly equipotent in squirrel monkey plasma (IC_{50}, 1.0 nmol/L). Dog and guinea pig plasma renin were also inhibited reasonably with IC_{50} values of 29 and 65 nmol/L, respectively, but rat plasma renin was inhibited only in the micromole per liter range.

We tested the principal hemodynamic effects in sodium-depleted squirrel monkeys. Intravenous administration of the relatively high dose of 0.1 mg/kg led to a decrease of mean arterial pressure by 20 to 30 mm Hg without a change in heart rate (Fig 2). The duration of blood pressure decrease was long, exceeding 8 hours.

We tested oral activity first acutely in both sodium-depleted normotensive squirrel monkeys (Fig 3A) and squirrel monkeys made hypertensive by chronic treatment with cyclosporin A (Fig 3B). In both models ciprokiren showed dose-dependent potent oral activity, with significant blood pressure lowering even at the low oral dose of 3 μg/kg. A maximal blood pressure lowering of 40 mm Hg was reached with 3 mg/kg in the normotensive monkeys and with 0.3 mg/kg in the hypertensive monkeys, respectively. Compared with remikiren, ciprokiren showed a 10-fold higher oral potency, as the same blood pressure drop was reached with a 10-fold lower dose (Fig 4). However, the intrinsic efficacies of remikiren and ciprokiren were similar, as administration of supramaximal oral doses (10 mg/kg) of one inhibitor on top of the other did not lead to a further decrease of arterial blood pressure (Fig 5A and 5B).
decrease of mean arterial pressure, which reached its nadir on the seventh day (Fig 6). In contrast, in the placebo-treated monkeys, blood pressure was unchanged during the entire duration of the experiment.

A similar blood pressure reduction could be reached when ciprokiren was applied transdermally (Fig 7). When the patch comprising a formulation of ciprokiren was applied, blood pressure was again reduced progressively in the hypertensive monkeys. The effect was maximal after 7 days and on removal of the patch returned slowly within days to normal.

Finally, we studied the effects of ciprokiren on the plasma RAS in sodium-depleted squirrel monkeys using a dose that maximally reduced blood pressure for the entire experimental period (Fig 8). Oral administration of 10 mg/kg led to the expected immediate changes of the different variables. At 30 minutes, PRA and immunoreactive Ang II were significantly suppressed by 65% and 42%, respectively, compared with the predrug levels in the ciprokiren group. In contrast, there was no reduction in the control group. It is worth noticing that neither PRA nor immunoreactive Ang II was completely reduced. Renin measured by its immunoreactivity was increased to 480%. Interestingly, after 4 hours, PRA levels were back to predrug values, but immunoreactive Ang II and blood pressure were still low, with increased immunoreactive renin. A similar evaluation in the transdermal experiment did not show any changes in PRA or immunoreactive Ang II in both the placebo and drug-treated groups (Fig 9) under conditions that effectively lowered blood pressure (Fig 7).

Discussion

The present results show that ciprokiren is a very potent inhibitor of human renin and is sixfold more
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potent than remikiren (IC50, 0.41 nmol/L in vitro and 3.0 nmol/L in human plasma). Interestingly, in human plasma it was 10-fold less active than in the in vitro test using pure renin and synthetic tetradecapeptide renin substrate instead of angiotensinogen. The result most probably reflects extensive plasma protein binding, which reduces the concentration of available free drug and thus results in a higher IC50 value, which is different from the true affinity. Indeed, such shifts in IC50 values have been observed for other renin inhibitors and seem to be species dependent. Since the inhibition of plasma renin was estimated in conditions as close as possible to physiological by the so-called trapping assay, which was also used for PRA estimations, the plasma IC50 value measured by this methodology should represent the “affinity” that is relevant for the plasma effects in vivo.

The selectivity of the compound is similar to that observed for most other renin inhibitors described and thus is not astonishing. It discriminates renin from the closely related enzymes cathepsin D and pepsin by more than 100 000-fold. Ciprokiren is relatively specific for primate renin because it inhibits plasma renin from other species to a lesser extent. This excludes the rat as a test model but offers the possibility of studying the compound in dogs and guinea pigs in addition to primates.

Ciprokiren potently reduces blood pressure without increasing heart rate. This absence of reflex tachycardia is typical for RAS blockers and may be explained by a suppression of the facilitatory effect of Ang II on the baroreceptor reflex. In contrast, peripheral vasodilators produce strong tachycardia in our models. Thus, this absence of tachycardia is a hint that ciprokiren indeed reduces blood pressure by renin inhibition and not by a nonspecific mechanism. This is confirmed by the fact that ciprokiren has the same efficacy as remikiren. Moreover, in marmosets we found that binephrectomy completely blunted the blood pressure reduction by 1 mg/kg IV ciprokiren (data not shown) in a manner similar to that described before for remikiren, which may give an additional hint for the specificity of ciprokiren in vivo.

After acute oral administration ciprokiren reduced arterial blood pressure similarly to remikiren but was 10-fold more potent. It is interesting to note that the difference between ciprokiren and remikiren in vitro is reflected also in the oral potency, suggesting that the pharmacokinetic behavior of both inhibitors is similar in our models.

Some insights on the mode of action of ciprokiren are offered by the estimation of the plasma RAS variables. Maximal blood pressure reduction was not paralleled by maximal reduction in PRA and immunoreactive Ang II. However, it is worth mentioning that the actual PRA inhibition is greater than that estimated by the comparison with the predrug value since plasma renin is increased because of the renin release (ie, at 30 minutes: 93% versus 65% inhibition when compared with the actual renin concentration or to predrug PRA, respectively). Possible hypotheses are that (1) no more reduction of these RAS variables is needed for a maximal fall in blood pressure, assuming that the plasma variables determine blood pressure, or (2) maximal reduction in renin and Ang II is achieved in an extraplasmatic site, suggesting that the active compartment would be in tissue and not in plasma. Interestingly, 4 hours after drug administration, PRA was back to
predrug levels, but blood pressure was still maximally decreased. This suggests that renin is inhibited in an extraplasmatic site and that the second hypothesis supporting an extraplasmatic site of action for ciprokiren may be assumed.

In this context it is important to note that we used a PRA methodology that should avoid measurement artifacts. For PRA estimation (and the estimation of plasma renin in vitro) we used the trapping methodology of Poulsen and Joergensen because this technique avoids the addition of angiotensinase inhibitors such as hydroxyquinolone, phenylmethylsulfonyl fluoride, or diisopropyl fluorophosphate to the plasma but protects Ang I from degradation by the addition of an excess of Ang I antibodies during the in vitro production of Ang I. The in vitro condition for the generation of Ang I is thus close to the physiological condition in vivo and allows for protein binding, which in turn reduces the available free concentration of renin inhibitor and therefore its inhibitory effects in vivo. Indeed, discrepant results have been obtained when the plasma of renin inhibitor–treated patients was analyzed by the trapping and angiotensinase inhibitor methodologies. Since the trapping but not the angiotensinase inhibitor PRA gave parallel results to Ang I and Ang II, we concluded that this methodology was the most relevant.

In contrast to renin inhibition in humans, plasma immunoreactive Ang II did not parallel the time course of PRA inhibition in our sodium-depleted squirrel monkeys after ciprokiren administration. The reason may be that in squirrel monkeys a major portion of plasma Ang I is generated by extraplasmatic renin, which is inhibited longer than plasma renin. However, extraplasmatic functional renin was shown to contribute to the plasmatic Ang I pool also in hypertensive patients. Finally, the reduced immunoreactive Ang II measured here is unlikely to be artifactual because it was associated with an increased renin release known to be caused by the interruption of the negative feedback mechanism by the reduction of Ang II. Similar results have previously been shown for remikiren in this squirrel monkey model.

In the transdermal experiment no changes of PRA and immunoreactive Ang II could be detected even when blood pressure was maximally reduced after 1 week. This absence of biochemical plasma effects could be expected, considering the low skin fluxes measured in vitro (see “Methods”), which cannot lead to sufficiently high plasma concentration. We tried to measure the plasma concentration by a bioassay at the fifth day and found less than 1 nmol/L ciprokiren (data not shown). Considering the specific mode of action of ciprokiren as discussed before, one had to assume a mere tissular effect induced by the upconcentration of ciprokiren in tissue. Indeed, preliminary pharmacokinetic measurements in rats showed a large volume of distribution of 2.8 L/kg, pointing to an extensive tissue distribution of ciprokiren.

More hints for an important extraplasmatic RAS and its variable inhibition by different renin inhibitors have been shown recently. Similar reductions in plasma immunoreactive Ang II by different renin inhibitors did not result in the same blood pressure decreases in sodium-depleted squirrel monkeys. Furthermore, in cyclosporin-induced hypertensive squirrel monkeys, neutralizing renin antibodies did not lead to a blood pressure decrease in contrast to remikiren, even though plasma immunoreactive Ang II was reduced similarly. Thus, in our models not even plasma immunoreactive Ang II was a correct indicator for blood pressure, which thus might be determined mainly via a tissular compartment. A similar conclusion might be drawn from experiments in binephrectomized cynomolgus monkeys, in which another renin inhibitor, enalikiren, was still able to reduce blood pressure. However, these results are in contrast with ours and might be due to the high doses of enalikiren used or to technical differences. Finally, in hypertensive patients remikiren reduced blood pressure persistently, whereas plasma Ang II was reduced only transiently. Similarly, enalikiren was shown to have blood pressure effects dissociated from its actions on the plasma RAS.

More experimental findings indicate the importance of extraplasmatic Ang II. In spontaneously hypertensive rats, chronic ACE inhibition seemed to lead to a reversal of initially lowered Ang II levels under still reduced arterial pressure. On the other hand, in the same model administration of neutralizing monoclonal Ang II antibodies led to reduced Ang II pressor response but not to blood pressure reduction. And last, inserting the mouse Ren-2 gene in rats created fulminant hypertension without increased PRA or plasma Ang II.

The very long blood pressure decrease induced by ciprokiren might be caused by a long-lasting effect of the drug in important tissue compartments because it could not be related to plasmatic inhibition of renin. Based on this assumption, an accumulation of the drug with chronic treatment may have been expected to lead to progressive blood pressure reduction. Since extremely small oral doses were so effective, we applied a transdermal patch of ciprokiren. Indeed, this formulation led to a progressive blood pressure decrease similar to that with the small oral doses. After removal of the patch, it took several days for blood pressure to be recovered. Again, a possible explanation for this phenomenon might be the long tissular half-life of ciprokiren.

In conclusion, we have shown that ciprokiren is an extremely potent and orally active renin inhibitor with long-lasting effects. The full efficacy reached with very small doses given chronically either per os or transdermally indicates that such a dosage regimen should be considered for clinical studies with this type of drug.

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