Renin belongs to the A1 family of aspartic proteases. Its 3-dimensional (3D) structure consists of 2 β-sheet domains (N and C domain) related by an ≈2-fold axis. The active site is a deep cleft between the N and C domains that extends over 8 residues of renin’s substrate, angiotensinogen. Each domain supplies 1 catalytic aspartic acid residue in the center of the binding cleft. A long β-hairpin loop structure called the flap in the N-terminal domain covers the central part of the angiotensinogen-binding site, and the conformation with the tip of the flap open allows the entrance of the substrate and removal of hydrolytic products during the catalytic turnover, while the tip closes on substrate binding. In the case of renin’s inactive precursor, prorenin, the flap is open to accommodate the 43-amino acid prosegment. Binding to prorenin in the open conformation, as well as to the intermediate form of prorenin.3,4 Binding to the intermediate form induces prorenin unfolding. Because of the tight binding of the renin inhibitor, the refolding step (ie, the return to the closed conformation) is no longer possible, and thus the equilibrium between the closed and open conformation will shift in favor of the open conformation. Eventually, depending on the concentration of aliskiren, a significant proportion of prorenin may be open (nonproteolytic activation), allowing its recognition by the active site-directed antibodies used in renin immunoradiometric assays (IRMAs), despite the fact that the

**Key Words:** conformational changes • hypertension • prorenin • renin • renin inhibitor

Prosegment unfolding occurs in a pH- and temperature-dependent manner and, if not followed by cleavage, results in 2 prorenin conformations as follows: a closed, inactive form, and an open form that displays full enzymatic activity (Figure 1).3,4 In addition, an intermediate form exists where the prosegment has moved away from the cleft, but where the renin part still has to undergo the above-mentioned conformational changes. Under physiological conditions, <2% of prorenin is in the open conformation. The recently introduced renin inhibitor, aliskiren (Figure S1A in the online-only Data Supplement), binds to prorenin in the open conformation, as well as to the intermediate form of prorenin.3,4 Binding to the intermediate form induces prorenin unfolding. Because of the tight binding of the renin inhibitor, the refolding step (ie, the return to the closed conformation) is no longer possible, and thus the equilibrium between the closed and open conformation will shift in favor of the open conformation. Eventually, depending on the concentration of aliskiren, a significant proportion of prorenin may be open (nonproteolytic activation), allowing its recognition by the active site-directed antibodies used in renin immunoradiometric assays (IRMAs), despite the fact that the

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**New Renin Inhibitor VTP-27999 Alters Renin Immunoreactivity and Does Not Unfold Prorenin**

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**Abstract**—Renin inhibitors like aliskiren not only block renin but also bind prorenin, thereby inducing a conformational change (like the change induced by acid) allowing its recognition in a renin-specific assay. Consequently, aliskiren can be used to measure prorenin. VTP-27999 is a new renin inhibitor with an aliskiren-like IC_{50} and t_{1/2}, and a much higher bioavailability. This study addressed (pro)renin changes during treatment of volunteers with VTP-27999 or aliskiren. Both drugs increased renin immunoreactivity. Treatment of plasma samples from aliskiren-treated subjects with excess aliskiren yielded higher renin immunoreactivity levels, confirming the presence of prorenin. Unexpectedly, this approach did not work in VTP-27999–treated subjects, although an assay detecting the prosegment revealed that their blood still contained prorenin. Subsequent in vitro analysis showed that VTP-27999 increased renin immunoreactivity for a given amount of renin by ≥30% but did not unfold prorenin. Yet, it did bind to acid-activated, intact prorenin and then again increased immunoreactivity in a renin assay. However, no such increase in immunoreactivity was seen when measuring acid-activated prorenin bound to VTP-27999 with a prosegment-directed assay. The VTP-27999–induced rises in renin immunoreactivity could be competitively prevented by aliskiren, and antibody displacement studies revealed a higher affinity of the active site-directed antibodies in the presence of VTP-27999. In conclusion, VTP-27999 increases renin immunoreactivity in renin immunoassays because it affects the affinity of the active site-directed antibody. Combined with its lack of effect on prorenin, these data show that VTP-27999 differs from aliskiren. The clinical relevance of these results needs to be established. (Hypertension. 2013;61:1075-1082.) • Online Data Supplement
prosegment is still present and aliskiren is bound to the active site.\(^\text{4,5}\) Because prorenin levels are, on average, \(\approx 10\)-fold higher than those of renin, this may affect immunoreactive renin measurements in patients taking aliskiren.\(^\text{7,8}\) A direct prorenin ELISA, based on the recognition of an epitope near prorenin’s putative cleavage site, helps to circumvent this problem.\(^\text{9}\) This assay also detects the intermediate form of prorenin but not the open, active form.

VTP-27999 (Figure S1B) is a new, active site-directed renin inhibitor with an \(IC_{50}\) (0.3 nmol/L) and half life (30 hours) that are comparable to those of aliskiren (0.6 mmol/L and 40 hours, respectively), but with a much higher bioavailability (>20% versus 2.6%).\(^\text{5}\) This study tested the changes in renin and prorenin during a 10-day treatment of salt-depleted volunteers with 150 mg VTP-27999, focusing on the possibility that the VTP-27999-induced rise in renin is attributable, at least in part, to prorenin unfolding. Remarkably, this turned out not to be the case. Therefore we also performed an extensive biochemical analysis of the effects of VTP-27999 on renin and prorenin in vitro. A direct comparison with aliskiren revealed that both drugs have a different mode of action. To what degree this has clinical relevance remains to be determined.

**Methods**

**Studies in Humans**

Healthy volunteers (9 men, 1 woman; age 19–41 years) received a low-salt (10 mEq/d) diet for 5 days and were then given 150 mg VTP-27999 (\(n=6\)), 300 mg aliskiren (\(n=2\)), or placebo (\(n=2\)) once daily for 10 days, followed by a washout period of 72 hours, while continuously maintaining the low-salt diet. EDTA plasma was collected at various time points on day 1, 2, 10, 11, 12, and 13. Written, informed consent was obtained from each volunteer, and the protocol was approved by the PRACS Institute, Ltd, Institutional Review Board, Fargo, ND.

**Studies in Plasma and Buffer**

To study whether VTP-27999 affects the measurement of renin or prorenin, recombinant human renin and prorenin (0.19 and 0.10 mg/mL, respectively), a gift from Actelion Pharmaceuticals, Alschwil, Switzerland) were diluted in human plasma or phosphate buffer (0.0122 mol/L NaHPO\(_4\)·2H\(_2\)O; 0.0867 mol/L Na\(_2\)HPO\(_4\); 0.0759 mol/L NaCl; pH 7.4) containing 0.1% BSA (Sigma) and incubated at 4, 22, or 37°C in the absence or presence of VTP-27999 (at concentrations ranging from 0.001 to 100 μmol/L) for various time periods, after which renin, open prorenin, and total prorenin were measured. For comparison, similar studies were performed with aliskiren, either alone or on top of VTP-27999, and in plasma containing endogenous renin and prorenin, both before and after activating prorenin with trypsin.

Next, to address whether VTP-27999 binds to open, intact prorenin, recombinant human prorenin was fully unfolded (without cleaving off the prosegment) by incubating it in an acidic solution (0.05 mol/L glycine, 0.051 mol/L EDTA, 0.0049 mol/L NaCl; 0.1% BSA; pH 3.3) for 48 hours at 4°C.\(^\text{10}\) Thereafter, the samples were neutralized with ice-cold phosphate buffer containing 0.1% BSA in the absence or presence of VTP-27999 or aliskiren (at concentrations ranging 1–10 μmol/L), and incubation continued at 37°C for up to 1 hour, after which the assays discussed below were performed immediately at 4°C.

Finally, to investigate the possibility that VTP-27999 binding to renin affects the affinity of the monoclonal antibody recognizing renin’s active site, renin was determined at a fixed concentration (≈200 pg/mL) by renin IRMA with or without 0.1 μmol/L VTP-27999 in the presence of increasing concentrations (1 pmol/L to 1 μmol/L) of the nonlabeled active site-directed antibody R1-20-5.

**Biochemical Assays**

Renin was measured with the renin III (Cisbio, France) IRMA (detection limit 1 pg/mL). This assay, which makes use of a monoclonal antibody (4G1)\(^\text{11}\) directed against renin’s active site, also recognizes intact, open prorenin.\(^\text{3,12}\) This implies that intact prorenin can be measured with this assay after incubating it with acid or after exposing it, for 48 hours at 4°C, to 10 μmol/L aliskiren because both procedures induce the conversion of all prorenin molecules into the open conformation.\(^\text{6}\) Additionally, we converted prorenin to renin by cleaving off the prosegment with immobilized trypsin (72 hours at 4°C). In plasma, this approach yields identical total renin (renin+prorenin) levels as aliskiren exposure and thus, subtracting the renin levels measured before trypsin treatment or aliskiren exposure from those after these procedures indirectly provides an indication of the prorenin levels. (Figure 1)

Intact, closed prorenin was measured with an ELISA (detection limit 10 pg/mL) that recognizes residues 32 to 39 of the prosegment (Molecular Innovations, Novi, MI).\(^\text{6}\) This prorenin assay was performed according to the instructions of the manufacturer, making use of the above-mentioned human recombinant prorenin to construct the standard curve.

In a select set of samples, intact, open prorenin was measured on the basis of its prosegment, replacing the \(^{125}\)I-labeled active site-directed monoclonal antibody of the Cisbio kit by a progromment-directed \(^{125}\)I-labeled monoclonal antibody (F258-37-B1) in the IRMA (F258 IRMA; detection limit 10 pg/mL). F258-37-B1 is directed against the C-terminal part (p20–p43) of the propeptide and does not react (<0.1%) with renin. F258-37-B1 also does not
react (<0.1%) with intact, closed prorenin. However, it does react with prorenin after the above treatment of prorenin with aliskiren. Thus, the aliskiren-induced nonproteolytic conformational change, causing the propeptide to move to the surface of the molecule, allows the recognition of prorenin by both the active site-directed antibody of the Cisbio kit and the prosegment-directed antibody of the prorenin IRMA.

Finally, because VTP-27999 seemed to affect the outcome of the Cisbio IRMA, renin measurements in the presence of VTP-27999 were also performed with an alternative renin IRMA (Beckman Coulter, Immunotech, Prague, Czech Republic). This IRMA makes use of the active site-directed monoclonal antibody R1-20-5, and has a detection limit of 0.9 pg/mL.

Data Analysis
Results are shown as mean±SEM. Differences were tested using 1-way ANOVA, followed by Dunnett multiple comparison test; P<0.05 was considered significant.

Results
Measurements in Plasma of Placebo-, Aliskiren-, or VTP-27999–Treated Subjects
In placebo-treated subjects, plasma renin levels remained stable throughout the entire treatment period (Figure 2A). In vitro incubation of plasma with 10 μmol/L aliskiren resulted in a substantial rise in the immunoreactive renin levels detected by the renin IRMA. This is attributable to the fact that the assay now also recognizes prorenin (converted to an open, renin-like conformation by aliskiren; ie, it determines total renin). The difference between total renin (measured after aliskiren exposure) and renin (measured before aliskiren exposure) reflects the prorenin levels. Prorenin, like renin, remained stable in the placebo-treated subjects during the entire treatment period.

Aliskiren treatment acutely increased renin >20-fold on day 1, and further increases (up to 100-fold) were observed on day 10 (Figure 2B). Renin levels decreased with a half life of 39±10 hours after stopping treatment on day 10, in agreement with the half life of aliskiren. In vitro exposure to 10 μmol/L aliskiren yielded the total renin levels, and from these levels, prorenin could be calculated. As expected on the basis of its constitutive release, prorenin levels started to rise only after >12 hours and rose further during prolonged treatment. Prorenin levels had not yet returned to baseline before the end of the protocol (on day 13).

VTP-27999, like aliskiren, greatly increased renin levels on day 1, and further increases were observed on day 10 (Figure 2C). Renin levels decreased with a half life of 30±3 hours after stopping treatment, in agreement with the half life of VTP-27999. Unexpectedly, in vitro exposure to aliskiren of plasma samples, obtained from VTP-27999-treated subjects at ≥2 hours after VTP-27999 intake, did not yield total renin levels that were higher than the renin levels measured before aliskiren exposure. In some cases, the levels were even lower. On average, the levels were identical with and without aliskiren exposure. This was not the case in the samples taken during the first 2 hours after VTP-27999 intake nor in the samples taken after stopping treatment; in those samples aliskiren did increase the amount of immunoreactivity in the renin IRMA.

The lack of an aliskiren-induced rise in immunoreactive renin levels in samples from VTP-27999-treated subjects suggests that either prorenin had disappeared during such treatment or that VTP-27999 had already converted all prorenin molecules into an open conformation in vivo, leaving no additional effect to be induced by aliskiren in vitro. To investigate this, we measured intact, closed prorenin with the direct prorenin ELISA (Figure 2C). This assay clearly demonstrated the presence of prorenin during VTP-27999 treatment, behaving identically as after aliskiren treatment: a modest rise after >12 hours and a further rise at day 10, with no return to baseline yet on day 13. Moreover, the prosegment-directed F258 assay (which recognizes prorenin only when the prosegment has moved out of the active cleft, ie, in its open form) yielded no detectable prorenin levels in plasma...
samples of either placebo-treated (n=2) or VTP-27999-treated (n=5) subjects (data not shown). This confirms that plasma prorenin in VTP-27999-treated subjects was present in its closed conformation.

Additionally, we measured total renin in a subset of samples (obtained on day 10 between 4 and 6 hours after dosing) after incubating plasma samples of VTP-27999-treated subjects with immobilized trypsin to cleave off the prosegment. In these samples, immunoreactive renin levels rose from 1587±493 before, to 1813±524 pg/mL (n=6; P<0.005) after trypsin exposure. From these levels, plasma prorenin levels of 225±46 pg/mL could be calculated. These levels are ≈2-fold higher than the prorenin levels measured with the direct ELISA in the same samples (109±9 pg/mL; P<0.05).

Taken together, these data show that intact prorenin is still present after VTP-27999 but that the (indirect) aliskiren approach underestimates its levels, whereas the trypsin approach overestimates its levels.

Studies With Recombinant Human Prorenin

Next, we verified the effect of aliskiren and VTP-27999 on prorenin in vitro. Incubating recombinant human prorenin with aliskiren at increasing concentrations at 4°C for 48 hours resulted in prorenin unfolding, allowing its recognition in the renin IRMA (Figure 3A; n=4; pEC50≈6.2±0.02 [pEC50 indicates the negative logarithm of the inhibitor concentration at which 50% of the maximum effect has been reached]). In agreement with previous studies,5,6 10 μmol/L aliskiren was sufficient to fully unfold all prorenin molecules. Remarkably, incubation with VTP-27999 did not result in prorenin unfolding (n=4). Yet, a 24-hour preincubation at 4°C with VTP-27999 shifted the aliskiren unfolding curve ≈10-fold to the right (n=4; pEC50≈5.4±0.04; P<0.005 versus without VTP-27999). This illustrates that VTP-27999 and aliskiren display competition for the same binding site. When performing the reverse experiment (a 24-hour preincubation with aliskiren followed by a 48-hour incubation with VTP-27999), VTP-27999 unexpectedly increased the amount of immunoreactivity in the Cisbio assay by ≈30% in a concentration-dependent manner (n=4; pEC50≈5.4±0.1).

To determine whether temperature or plasma components are important determinants of prorenin activation by VTP-27999, we also studied unfolding at 0.1, 1, and 10 μmol/L VTP-27999 in human plasma at 4, 22, and 37°C, using 10 μmol/L aliskiren as a positive control (n=3 for each condition). Yet, again, no effect of VTP-27999 was seen (Figure 3B and 3D). In agreement with this observation, incubation of prorenin for 48 hours at 4°C with VTP-27999 (1 nmol/L to 100 μmol/L) also did not allow its detection in the F258 assay (n=3, data not shown).

Thus, both aliskiren and VTP-27999 bind to prorenin, but only aliskiren is capable of inducing prorenin unfolding.

Studies With Acid-Activated Prorenin

Subsequently, we activated (ie, unfolded) prorenin with acid.10 This procedure converts all prorenin molecules into the open, active conformation, allowing their detection in the renin IRMA (Figure 1). Neutralization will result in rapid refolding of prorenin so that it can no longer be detected. Thus, by measuring acid-activated prorenin in the renin IRMA at various time points after neutralization, one gets an indication of the velocity of the refolding process. At 37°C, open prorenin returned to its closed, inactive conformation with a t1/2 of 10±1 minutes (Figure 4A and 4B; n=4). This will not occur when locking prorenin in the open conformation, for example, because of the binding of a renin inhibitor to the active site (Figure 1). Indeed, both aliskiren and VTP-27999 prevented the refolding process in a concentration-dependent manner, and at concentrations of ≥100 μmol/L, both drugs fully locked prorenin in its open conformation (Figure 4A and 4B; n=3–4 for each concentration). Their affinities for open prorenin were identical (Figure 4C; pIC50≈8.2±0.1 versus 8.4±0.1). These data indicate that VTP-27999 is capable of binding to prorenin’s active site when it is exposed, and that once bound, it keeps prorenin in the open, unfolded conformation.

Unexpectedly, as demonstrated in Figure 4B, VTP-27999 not only blocked prorenin refolding but also increased the absolute levels detected in the renin IRMA by 41±7%.

To investigate the latter in further detail, we applied the renin IRMA to a fixed concentration of prorenin, either
Krop et al

VTP-27999 Differs From Aliskiren

1079

without pretreatment, after incubation with trypsin, 10 μmol/L aliskiren or 10 μmol/L VTP-27999, or after acid pre-treatment followed by neutralization in the presence of aliskiren or VTP-27999 (both 10 μmol/L). As expected, trypsin, aliskiren alone, and acid+aliskiren yielded the same maximum renin immunoreactivity level, and VTP-27999 alone did not unfold prorenin (Figure 4D; n=3 for each condition). Acid+VTP-27999 (n=3) again resulted in the detection of 40% more renin immunoreactivity, similar to the increases observed in Figures 3A and 4B. This suggests that VTP-27999 binding to the active site of unfolded prorenin either allows the detection of a prorenin variant that previously remained undetected (thus resulting in higher renin levels) or affected the binding affinity of the active site-directed antibody of the renin IRMA. To verify the former, we also measured acid-activated prorenin on the basis of its prosegment (with the F258 assay) in the presence of 100 nmol/L VTP-27999. This assay did not reveal an increase in immunoreactivity after VTP-27999 (98±4 versus 117±11 pg/mL in the presence of 10 μmol/L aliskiren; n=4), thereby excluding the concept that we detected a new prorenin variant with the renin IRMA that could previously not be detected.

In summary, both VTP-27999 and aliskiren lock unfolded prorenin in its open conformation, allowing its detection in a renin IRMA. VTP-27999 binding causes an additional rise in renin (but not prosegment) immunoreactivity, most likely because such binding selectively affects the affinity of the active site-directed antibody.

Studies With Renin

To verify whether VTP-27999 affected the detection of renin, we quantified recombinant human renin in phosphate buffer in the presence of increasing concentrations of VTP-27999. When present during the assay procedure, VTP-27999 concentration dependently increased the amount of renin detected in the renin IRMA (Figure 5A; n=3), until at VTP-27999 concentrations of ≥10 nmol/L, consistently 30% to 40% higher renin levels were detected, identical to the rise observed in Figure 4D for acid-activated prorenin. Such increases were not observed with aliskiren at concentrations up to 1 μmol/L. Importantly, when preincubating renin in buffer with VTP-27999 (0.1, 1, or 10 μmol/L; n=4 for each condition) for 60
minutes at room temperature and then performing the renin assay in the presence of increasing aliskiren concentrations, it became clear that aliskiren annihilates the effect of VTP-27999 in a concentration-dependent manner (IC_{50}, 0.3±0.2, 1.8±0.5, and 21±7 µmol/L, respectively; Figure 5B).

Given the above findings on recombinant human renin, we also wanted to know whether this applies to endogenous renin. To this end, plasma samples of nonrenin inhibitor-exposed subjects (n=5), before and after trypsin activation, were measured in the presence of increasing concentrations of VTP-27999. Similar to recombinant human renin, maximum increases in renin immunoreactivity were observed at VTP-27999 concentrations of ≥10 µmol/L. Before trypsin, these increases amounted to maximally 30±3 pg/mL (+70±6%, whereas after trypsin, these increases amounted to 48±4 pg/mL (+35±1%; Figure 5C; P<0.05 versus before trypsin). The larger absolute rise after trypsin indicates that the increase in renin immunoreactivity involved both renin and trypsin-activated prorenin. Like in buffer, the VTP-27999 (1 µmol/L)-induced rise in renin immunoreactivity could be fully reversed by aliskiren (IC_{50} 0.8±0.2 µmol/L; Figure 5D). Furthermore, aliskiren (0.01–100 µmol/L) decreased the level of renin immunoreactivity in 4 trypsin-pretreated plasma samples of VTP-27999–treated subjects, taken between 4 and 6 hours after oral dosing on day 10, by maximally 27±1% (data not shown).

In summary, VTP-27999 binding to renin increases renin immunoreactivity by ≥30%, and aliskiren can annihilate this effect in a competitive manner. Thus, both inhibitors compete for the same binding sites, but only VTP-27999 binding affects the affinity of the active site-directed antibody. Not surprisingly, this phenomenon also applies to trypsin-cleaved prorenin. These observations imply that the renin levels in plasma samples of VTP-27999–treated subjects may have been overestimated by ≥30%. Preincubation of such samples with high aliskiren levels (100 µmol/L) prevents this phenomenon.

**Does VTP-27999 Alter the Affinity of the Active Site–Directed Antibodies?**

Making use of a renin IRMA (Beckman Coulter) that applies a different active site-directed antibody (R1-20-5 versus 4G1 in the Cisbio IRMA; Figure 1), we were able to confirm the increase in renin immunoreactivity after VTP-27999 exposure, both in the case of acid-activated prorenin (Figure 6A; n=3) and renin (Figure 6B; n=2). Moreover, in the presence of VTP-27999, it took 4-fold higher R1-20-5 concentrations to prevent the binding of 125I-labeled R1-20-5 (Figure 6C; IC_{50} 7.7±0.01 versus 7.1±0.01; n=3) or 125I-labeled 4G1 (Figure 6D; IC_{50} 7.3±0.03 versus 6.7±0.01; n=3) than in the absence of VTP-27999. Thus, VTP-27999 binding to the active site increases the affinity of these 2 active site-directed antibodies. This explains the higher immunoreactivity levels of acid-activated prorenin and renin in the presence of VTP-27999 (Figures 4D, 5A, and 5C).

**Discussion**

This study shows that the renin inhibitor VTP-27999 acts differently from aliskiren. First, VTP-27999 binding to renin increases renin immunoreactivity in 2 different renin IRMAs.

Second, in contrast to aliskiren (and to other renin inhibitors, including remikiren),5 6 VTP-27999 does not induce prorenin unfolding. It does, however, bind to acid-activated, open prorenin, and by doing so, it keeps prorenin in this open conformation and then again increases the level of renin immunoreactivity when measuring this open prorenin in a renin IRMA.

Adding VTP-27999 at concentrations of ≥10 mmol/L during the renin IRMA of plasma samples increased the detected amount of immunoreactive renin by ≥30%. Similar increases were observed when measuring recombinant human renin in buffer. Aliskiren was capable of preventing the VTP-27999 effect in a concentration-dependent manner. This demonstrates that VTP-27999 and aliskiren compete for the same binding site, but that only VTP-27999 binding produces a surface
that increases the affinity of the active site–directed antibody. Interestingly, on the basis of crystallization studies, Rahuel et al.\textsuperscript{14} have proposed the existence of 2 renin conformations: an open, uninhibited conformation, and a closed, inhibited form. In the closed structure, the C-terminal loop segments forming the active site are closer to those from the N-terminal domain than in the open structure. Crystallographic analysis of the binding of aliskiren and VTP-27999 to these 2 forms reveals that the 2 inhibitors bind to both conformations, and that on binding, the flap is closed (Figures S2–S4). In the case of VTP-27999, the conformations of the 2 inhibited monomers are nearly identical, whereas with aliskiren, the 2 monomers differ from one another by the movement of the C-terminal domain closing down over the aliskiren-filled binding pocket. To what degree these conformational differences would alter the affinity of the active site-directed antibodies is unknown. Unfortunately, the binding epitopes of the 2 active site–directed monoclonal antibodies applied in our study (which were generated >30 years ago) are unknown. Nevertheless, our data show clearly that, in the presence of VTP-27999, more unlabeled antibody is required to displace the \textsuperscript{125}I-labeled active site-directed antibody, thereby demonstrating that binding occurs with higher affinity in the presence of VTP-27999.

The VTP-27999–induced increase in renin immunoreactivity also occurred when measuring trypsin-activated prorenin and ac-id-activated prorenin. The former is not surprising because trypsin converts prorenin to renin. The latter is in agreement with the observation that acid-activated, unfolded prorenin has the same 3D conformation as renin (and displays full enzymatic activity),\textsuperscript{10,15} although it still contains the prosegment. The presence of the prosegment allowed us to measure VTP-27999-bound, unfolded prorenin also with a second antibody directed against the prosegment (F258 IRMA, Figure 1). With this antibody, no increase in immunoreactivity was observed in the presence of VTP-27999. Therefore, it seems that VTP-27999 affected the binding of the active site-directed antibodies only.

The conversion of closed, inactive prorenin to open, active prorenin is a 2-step process. First, the prosegment moves out of the cleft, resulting in an intermediate form that cannot be detected in a renin IRMA and that does not display activity. Next, this intermediate form undergoes the required conformational changes making it indistinguishable from renin. Aliskiren and other renin inhibitors bind to the intermediate form and subsequently induce prorenin unfolding. Because of their tight (almost irreversible) binding, the equilibrium will shift into the direction of the open form.\textsuperscript{3,4} To fully convert all prorenin molecules into the open form, aliskiren concentrations of 10 μmol/L are required.\textsuperscript{5} Such high concentrations do not occur in vivo during aliskiren treatment.\textsuperscript{16} Nevertheless, even if only a small percentage of prorenin obtains the open conformation in vivo during aliskiren treatment, this may already result in an overestimation of the renin rise during renin inhibition.\textsuperscript{4,7,8} Our current data show that ex vivo exposure of plasma samples obtained from aliskiren-treated subjects to 10 μmol/L aliskiren still led to further rises in immunoreactive renin. Thus, indeed not all prorenin molecules had already obtained the open conformation in vivo during aliskiren treatment, in full agreement with previous studies.\textsuperscript{4,7,8} Yet, ex vivo exposure of plasma samples obtained from VTP-27999–treated subjects to aliskiren did not result in a further rise in immunoreactive renin, and in some samples even a decrease occurred.

At first sight, this suggests that VTP-27999, in contrast to aliskiren, had already converted all prorenin molecules into the open conformation in vivo. However, we were unable to detect unfolded prorenin (with the F258 assay) in plasma samples of VTP-27999–treated subjects. Moreover, in vitro studies revealed that VTP-27999 is incapable of inducing prorenin unfolding.

An explanation of these findings can now be derived from our observations on immunoreactive renin after its incubation with VTP-27999. VTP-27999 acutely increases the results of the renin assay by 230%, and aliskiren (partially) blocks this effect. Thus, at renin levels that are ≥2 orders of magnitude above normal (and 10-fold higher than renin; Figure 2C), VTP-27999 will yield an increase in renin immunoreactivity that is far higher than the actual prorenin levels (eg, at a renin level of 1000 versus 100 for prorenin, the renin assay will detect 1300). Even when aliskiren fully displaces VTP-27999 from renin, thereby diminishing its increasing effect (ie, the renin level goes back from 1300 to 1000), this will not allow us to detect the simultaneously occurring (much smaller!) increase in immunoreactivity attributable to aliskiren-dependent prorenin activation (+100 in the above example, resulting in a total renin level of 1100 after aliskiren versus 1300 before aliskiren). Consequently, depending on the actual VTP-27999 levels (>30 [trough]–1300 [peak] nmol/L; R. Gregg, unpublished data), the renin rise and the prorenin levels, both rises and decreases in renin immunoreactivity may be expected after aliskiren exposure in vitro. This is exactly what happened. Thus, in samples of VTP-27999–treated subjects, prorenin can only be detected indirectly using the trypsin method or directly by measuring prorenin with a direct prorenin assay. The results of the latter assay were lower than when applying the renin IRMA after trypsin exposure. This is because of the fact that VTP-27999 will also affect the detection of renin generated by trypsin from prorenin. Therefore, the best way to measure prorenin during VTP-27999 treatment is by making use of the direct prorenin ELISA.

Importantly, although VTP-27999 did not induce prorenin unfolding, it blocked the aliskiren-induced unfolding of prorenin (Figure 3A). This implies that VTP-27999 apparently does bind to the intermediate form of prorenin but that such binding has no conformational consequences. An explanation for the different consequences of inhibitor binding is that aliskiren would be more effective than VTP-27999 at displacing the Ser3 to Asp11 segment from the binding site, which in turn pushes out the prosegment (Figures S5–S8).\textsuperscript{3,4,17,18} The stability of the prosegment conformation is only hinted at by the solution of the prorenin crystal structure (Figure S9), which provides a time- and space-averaged snapshot of the zymogen. Understanding the dynamics of this portion of the prorenin structure would shed light on how these particular inhibitors interact with the protein and vice versa.

**Perspectives**

VTP-27999 binding to renin increases its immunoreactivity in renin IRMAs, thereby resulting in an overestimation of the renin rise in subjects treated with VTP-27999 by ≈30%.
Such changes in immunoreactivity do not occur with aliskiren (Figure 5A). In addition, VTP-27999 does not induce prorenin unfolding and thus, unlike aliskiren, does not allow the detection of prorenin in a renin IRMA. Aliskiren competitively displaces renin-bound VTP-27999, thereby annihilating the rise in renin immunoreactivity, and allowing a true estimation of the renin rise. Yet simultaneously, aliskiren is capable of converting prorenin to its open, renin-like conformation, thereby resulting in the (indirect) detection of prorenin in renin IRMAs. As a consequence, in plasma samples of VTP-27999–treated subjects, aliskiren may both decrease and increase renin immunoreactivity, and prorenin cannot be determined reliably in such samples. To overcome this problem, a direct prorenin assay is required. Future studies should investigate whether these biochemical differences of VTP-27999 versus other renin inhibitors yield clinically relevant differences. For instance, the lack of effect on the 3D structure of prorenin may reduce the possibility that there is a pool of unfolded prorenin, which on renin inhibitor dissociation (eg, at trough levels of the drug) would display activity locally. Furthermore, the different binding profile of VTP-27999 may affect renin and prorenin’s affinities for their putative receptors, including the mannose 6-phosphate/insulin-like growth factor II receptor and the (pro)renin receptor.19,20

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Disclosures

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References


Novelty and Significance

What Is New?

- The renin inhibitor VTP-27999 increases renin immunoreactivity in renin immunoassays most likely because it affects the affinity of the active site-directed antibody.
- VTP-27999, unlike aliskiren and other renin inhibitors, does not unfold prorenin.

What Is Relevant?

- Not all renin inhibitors are equal. In addition to a much higher bioavailability, VTP-27999 induces biochemical alterations in renin and prorenin that are different from those of aliskiren. To what degree this translates into relevant clinical differences needs to be explored.

Summary

When measuring the changes in renin and prorenin after the application of the new renin inhibitor VTP-27999 to healthy volunteers, it became clear that this renin inhibitor not only increases renin immunoreactivity, because it interferes with the angiotensin II–renin negative feedback loop, but also because it affects the renin assay. Moreover, unlike other renin inhibitors, it does not affect the prorenin conformation. Future studies should now investigate the clinical relevance of these biochemical alterations, which distinguish VTP-27999 from other renin inhibitors.
New Renin Inhibitor VTP-27999 Alters Renin Immunoreactivity and Does Not Unfold Prorenin
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THE NEW RENIN INHIBITOR VTP-27999 ALTERS RENIN IMMUNOREACTIVITY AND DOES NOT UNFOLD PRORENIN

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SUPPLEMENTAL INFORMATION

Crystallographic studies in silico

**Aliskiren versus VTP-27999**

Aliskiren (Figure S1A) can be considered an inhibitor with an amino-alcohol warhead located near the center of the molecule so that it will interact with renin subsites on the primed and non-primed sides of the binding pocket. VTP-27999 (Figure S1B), on the other hand, uses a secondary amine warhead located at the very end of the molecule so that it only interacts with the non-primed subsites.

In the co-crystal structure of aliskiren bound to human renin solved by Rahuel et al. (RCSB Protein Data Bank accession number: 2V0Z.pdb), there are two monomers in the crystallographic asymmetric unit that differ from one another by the movement of the C-terminal domain closing down over the aliskiren-filled binding pocket (Figure S2A – the overlay of the two aliskiren-bound monomers in the crystallographic asymmetric unit; note the difference between the two monomers at the top right, red to green loop). The average root-mean-square (RMS) deviation between the Cα’s of monomer “A” and monomer “B” was calculated to be 0.490Å using the LSQ option in the molecular graphics program, Coot. Aliskiren is tucked into the binding pocket of the N-terminal domain and the “flap” (residues Leu73 to Val80) is found in the “closed” conformation, covering the amino-alcohol portion of the inhibitor near the center of the small molecule. Aliskiren occupies the renin subsites S3SP, S3, S1, S1’ and S2’, where S3SP is a special pocket that projects deeper into the hydrophobic core of the protein from the S3 subsite. Figure S2B is the overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and VTP-27999 (RCSB Protein Data Bank accession code: 3Q4B.pdb). In this case, the average RMS deviation between the Cα’s of monomer A and monomer B is calculated to be 0.193Å. The conformations of the two inhibited monomers are nearly identical. VTP-27999 occupies the renin subsites S3SP, S3, S2 (barely) and S1. VTP-27999 does not extend into the primed subsites, as noted above. The flap is also found in the closed conformation in this crystal structure.

Figure S2C depicts the overlay of the two monomers (P+A vs. Q+B) found in the asymmetric unit of the crystal structure of human prorenin (RCSB Protein Data Bank accession code: 3VCM.pdb). Here the flap is found in the open conformation for monomer Q+B and in a more extended open conformation for monomer P+A. The average RMS deviation between the Cα’s of monomer P+A and monomer Q+B is 0.834Å, the largest difference of the three pairs.

Figure S3A is a close-up of aliskiren bound to monomer A (2V0Z.pdb) of human renin and shows a number of H-bonds (green, dashed lines) between protein and inhibitor, especially between the warhead atoms, N22 and O24 and the side chains of Asp32 and Asp215 and the carbonyl of Gly217. The ether oxygen, O2, is H-bonded to the amide nitrogen of Tyr14 in the special pocket, S3SP. Also, the two prime-side residues of aliskiren set up a short antiparallel beta-sheet with local parts of the protein. On the other hand, in the close-up of VTP-27999 bound to monomer A (3Q4B.pdb – Figure S3B), most of the H-bonds between VTP-27999 and the protein are between the warhead atom, N1 and the active site aspartic acids, Asp32 and Asp215. There are two additional H-bonds between VTP-27999 and flap residues Ser76 and Thr77, two more between the carbonyl oxygen of Gly217 and N2 and N4 of the inhibitor and one at the bottom of the special pocket, S3SP, between the amide nitrogen of Tyr14 and O3 of the carbamate. Figure S4A shows the overlay of these two inhibited structures. The RMS deviation for all Cα’s is 0.316Å between these two A monomers. Figure S4B is a close-up of the renin active site of the overlaid structures. Notice how the left hand side of the two inhibitors interact with the enzyme; aliskiren goes from subsite 1 directly to subsite 3, while VTP-27999 passes through or close to subsite 2 before dropping into subsite 3.
Inhibited structures versus prorenin
Figure S5 is the overlay of prorenin monomer A with the monomer A’s of the two inhibited crystal structures. The flap in the prorenin structure is in an extreme open conformation compared to either of the inhibited structures as a direct result of prosegment residue Met41P and mature renin residues Thr7 and Tyr9 pushing into the area between the flap and the N-terminal domain. The tetrahydropyrane on VTP-27999 also starts to push against flap residues, while aliskiren behaves like a peptidomimetic and allows the flap to close. The two inhibitors follow the trajectory of the prorenin mature chain segment bound in the active site. In Figure S6A it is clear that aliskiren would compete with this segment Val4 to Tyr9. VTP-27999, on the other hand, would directly compete with Leu6 to Tyr9 and maybe the main chain nitrogen of Met10; it would not interfere with binding to any of the prime-subsites (Figure S6B). This can be more clearly seen in Figures S7 (aliskiren versus protein segment Ser3 to Asp11) and S8 (VTP-27999 versus Ser3 to Asp11). These observations suggest that aliskiren would be more effective at displacing the Ser3 to Asp11 segment from the binding site which in turn pushes out the covering propeptide allowing for antibody detection of the exposed propeptide.

A last look at the prorenin structure
Morales et al.\(^3\) pointed out that the prosegment of the P+A monomer could be locked into the observed conformation by crystal lattice contacts, such as the stacking of Trp37P with a symmetry-related Arg74 sequestering the processing site of the prorenin P+A monomer (which, in solution, should actually be available). This might also be the reason that we see the prosegment of P+A folding over and sitting on the mature renin segment Val4 to Asn8. In Figure S9A, there is a large segment of the Q+B prosegment that is invisible to X-ray analysis because of its suspected mobility due to a lack of crystal lattice contacts as found with the corresponding P+A prosegment. Taking a closer look at the prosegment section that is visible and actually bound in the vicinity of the active site aspartates (Figure S9B), we see a residue “frame-shift” (i.e. compare Leu6A and Leu6B) and as we move from left to right, the prosegments start to go off in different directions. X-ray crystallography provides a time and space-averaged snapshot of the material trapped in a crystal. In this particular case, we have a clue to the dynamic nature of the prosegment of this zymogen by comparing the two monomers present in the crystallographic asymmetric unit. To determine the conformations of the prosegment that are possible in solution, we need to either determine them experimentally by a technique like NMR or in silico by running long time simulations using molecular dynamics. Knowing what conformations are available would result in a better understanding of the interactions of the two renin inhibitors with their prorenin/renin target.

References
Figure S1. A, The structure of aliskiren. B, The structure of VTP-27999.
Figure S2. A, The overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and aliskiren (2V0Z.pdb). The proteins are shown as Cα traces with aliskiren given in line format. Monomer A is green and the associated aliskiren molecule blue, while monomer B is colored red with its associated inhibitor colored yellow. B, The overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and VTP-27999 (3Q4B.pdb). Monomer A is blue with the associated VTP-27999 green, while monomer B is colored yellow with its associated inhibitor colored red. C, The overlay of the two monomers found in the asymmetric unit of the crystal structure of human prorenin (3VCM.pdb). Monomer A is cyan, while monomer B is colored magenta.
Figure S3. A, Aliskiren (magenta) binding site residues (monomer A). Hydrogen bonds (i.e. interaction distances between nitrogen and oxygen atoms between 2.4Å and 3.2Å) between aliskiren and renin are shown as green, dashed lines. Asp32 and Asp215 carbons are colored red. Residues with an atom within 3.5Å of aliskiren have yellow-colored carbons; within 4.0Å have cyan-colored carbons and within 5.0Å have white-colored carbons. B, VTP-27999 binding site (monomer A) with residues colored as above.
Figure S4. A, Overlay of monomer A from the co-crystal structure of the renin/aliskiren complex with monomer A of the renin/VTP-27999 complex. The protein in the two complexes is represented as a solid ribbon cartoon where the protein in the aliskiren complex is colored as red alpha helices, cyan beta sheet, and green turns and white random coils, while the protein in the VTP-27999 complex is colored yellow for contrast. The active site aspartic acids, Asp32 and Asp215 are shown colored red for the former and yellow for the latter complex. The two inhibitors are rendered in stick format where the carbon atoms of aliskiren are colored magenta and those of VTP-27999 are colored blue. Oxygens are red, nitrogens are gray and chlorine is colored green. B, A close-up of the active site with the flap residues removed for clarity.
Figure S5. A, Side view overlay of renin/aliskiren complex (2V0Z – monomer A – protein cartoon is colored as red alpha helices, cyan beta sheet, green turns and white random coils and stick aliskiren carbons are colored red), renin/VTP-27999 complex (3Q4B.pdb – monomer A – yellow protein and VTP-27999 with blue carbons) and prorenin (3VCM.pdb - monomer A – magenta protein and residue carbons). B, Close-up of the binding pocket.
Figure S6. A, Close-up of side view overlay of renin/aliskiren (rendered as in Figure S5) and prorenin (residues now shown with green carbons). B, Close-up of side view overlay of renin/VTP-27999 and prorenin.
Figure S7. Close-up of the overlay of renin/aliskiren (rendered as in Figure S5) and prorenin (residues shown with green carbons). A, Side view; B, top view.
**Figure S8.** Close-up of the overlay of renin/VTP-27999 (rendered as in Figure S5) and prorenin (residues shown with green carbons). A, Side view; B, top view.
Figure S9. A, Top view of the overlay of residues Thr6P to Glu17 for monomers P+A (magenta) and Q+B (yellow) after least squares superposition of Cα’s from prorenin polypeptide B against Cα’s of polypeptide A. The dashed curve connects Arg43P with Leu5 of the P+A prosegment. This cannot be done with the Met30Q to Val4 segment of the Q+B prosegment because of the absence of electron density between these two residues. The N and C termini of these segments are indicated. B, Close-up, side view of the residues between Val4 and Met10 following superposition described in A. Carbon atoms colored as in A.