In Vitro and in Vivo Characterization of the Activity of Telmisartan: An Insurmountable Angiotensin II Receptor Antagonist

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ABSTRACT

In vitro studies have shown that telmisartan is an insurmountable angiotensin II subtype-1 (AT1) receptor antagonist. Herein, the molecular basis of this insurmountable antagonism has been investigated in vitro, and the effect of telmisartan has been compared in vivo with that of irbesartan and candesartan. Association and dissociation kinetics of telmisartan to AT1 receptors have been characterized in vitro on rat vascular smooth muscle cells (RVSMC) expressing solely the AT1 receptor subtype. In a second set of experiments, the antagonistic efficacy of single intravenous doses (0.1, 0.3, and 1 mg/kg) of telmisartan was compared with that of irbesartan (0.3, 1.0, 3.0, and 10.0 mg/kg) and candesartan (0.3 and 1 mg/kg) in conscious, normotensive, male Wistar rats. The results show that the specific binding of [3H]telmisartan to the surface of living RVSMC is saturable and increases quickly to reach equilibrium within 1 h. Telmisartan dissociates very slowly from the receptor with a dissociation half-life (t1/2) of 75 min, which is comparable with candesartan and almost 5 times slower than angiotensin II (AngII). In vivo, telmisartan blunts the blood pressure response to exogenous AngII dose dependently. The blockade is long lasting and remains significant at 24 h at doses >0.1 mg/kg. Ex vivo assessment of the AT1 receptor blockade using an in vitro AngII receptor binding assay shows similar results. When administered intravenously in rats, telmisartan is 10-fold more potent than irbesartan and comparable to candesartan. Taken together, our in vitro data show that the insurmountable antagonism of telmisartan is due at least in part to its very slow dissociation from AT1 receptors.
been proposed including the presence of allosteric binding sites on the AT1 receptor (Timmermans et al., 1991; Wienen et al., 1992), a possible modification of the receptor or change in its conformation (De Chaffoy de Courcelles et al., 1986; Robertson et al., 1994), two antagonist-induced receptor states with fast and slow dissociation (Fierens et al., 1999b), the slow removal of the antagonist from tissue compartments, cells, or matrices surrounding the receptor (Panek et al., 1995), the coexistence of different subtypes of the AT1 receptor, or the ability of antagonists to modulate the amount of internalized receptors (Liu et al., 1992). Recently increasing evidence has been provided suggesting that a slow dissociation from the receptor resulting in an increased longevity of the antagonist-receptor complex is one of the leading mechanisms of the insurmountable characteristic of angiotensin II receptor antagonists (Fierens et al., 1999a; Vanderheyden et al., 2000a, b).

The purposes of the present study are: 1) to study the mechanisms of the insurmountable antagonism of telmisartan, and in particular, to determine the kinetics of association and dissociation of this drug to AT1 receptors; and 2) to compare in vivo the antagonistic activity of increasing doses of telmisartan with that of irbesartan and candesartan, two other long-lasting AT1 receptor antagonists.

Materials and Methods

Drugs

Telmisartan (Wienen et al., 1993), [3H]telmisartan (20 Ci/mmol), and irbesartan (Czaubon et al., 1993; Herbert et al., 1994) were obtained from Boehringer Ingelheim (Biberach, Germany). Candesartan was supplied by AstraZeneca Pharmaceuticals LP (Möndal, Sweden); [3H]candesartan (16 Ci/mmol) was provided by Amersham Biosciences (Piscataway, NJ), and 125I-AngII (angiotensin II (5-isoleucine/tyrosyl-labeled) was purchased from DuPont (Boston, MA). The other chemicals were of the highest grade commercially available.

Radioligand Binding Studies

The receptor binding assay was conducted either on intact rat smooth muscle cells solely expressing the angiotensin II AT1 receptor subtype (Burnier et al., 1995) or on membrane preparations obtained from the same cells.

Preparation of Rat aortic Smooth Muscle Cells

Membranes were obtained according to a method published previously (Maillard et al., 1998). For each experiment, an aliquot of membranes was thawed and centrifuged, and the pellet was resuspended in a 50 mM Tris-HCl, pH 7.2, containing 5 mM MgCl2 binding buffer to obtain a final concentration of 1 mg of protein/ml. The protein content of the final suspension was verified using the bicinchoninic acid protein assay (Pierce, Rockford, IL). The protein content of the final suspension was verified using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Angiotensin II Receptor Binding Assay on Rat Aortic SMC-Membrane Preparation

The binding of 125I-AngII (5 fmol/sample) or [3H]telmisartan (different concentrations) to RVSMC membranes (100 μg of protein/sample) was performed in a final volume of 400 μl of 50 mM Tris-HCl, pH 7.2, containing 5 mM MgCl2, the test compound, and 25 μl of rat plasma, resulting in a final percentage of 0.43% protein. A reference plasma, obtained by pooling the plasmas of several untreated animals, was used all along the study to standardize the results. After a 1-h incubation at 37°C, separation of bound labeled ligand was achieved by centrifugation, and residual radioactivity was determined by gamma counting (125I-AngII) or, for [3H]telmisartan, in 2 ml of a liquid scintillation cocktail (Hionic-Fluor; Canberra Packard, Groningen, The Netherlands) using a liquid scintillation counter. Nonspecific binding was estimated by adding 10 μM of unlabeled AngII or cold telmisartan to the incubation mixture. Specific binding was defined as total binding minus nonspecific binding.

Angiotensin II Receptor Binding Assays on Living Rat Aortic SMC

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Basle, Switzerland) with 15% fetal calf serum. Binding was performed 3 days after plating the cells on 24-well plates (Costar, Cambridge, MA). At confluence (ca. 200,000 cells/well equivalent to about 300 μg of protein/well), the cells were washed twice with 0.5 ml of DMEM/well at room temperature and left for 15 min at 37°C in 400 μl of DMEM. The experiments were started by replacement of DMEM by 400 μl of the assay buffer DMEM containing 10 mM Heps and 0.45% bovine serum albumin, pH 7.4. The binding of 125I-AngII (5 fmol/sample), [3H]telmisartan (different concentrations), or [3H]candesartan (different concentrations) was performed in 0.4 ml of assay buffer for 1 h at 37°C. The experiments were terminated by placing the cells on ice, aspirating the supernatant, and washing the cells three times with ice-cold binding buffer. The cell surface binding of [3H]telmisartan, [3H]candesartan, or 125I-AngII was extracted by mild acid treatment (i.e., a 5-min incubation with 0.5 ml of ice-cold 50 mM glycine buffer, pH 3, containing 125 mM NaCl). This step was repeated, and the radioactivity in the pooled fractions was counted after the addition of 10 ml of scintillation liquid cocktail (Hionic-Fluor) in a liquid scintillation counter for [3H]telmisartan and [3H]candesartan or directly by gamma counting (125I-AngII). To measure internalized radioligand (only for 125I-AngII binding), the cells were lysed with 0.1 M NaOH containing 2% Na2CO3 and 0.1% SDS, and the solubilized radioactivity was counted. This solution was also used for protein content determination. The nonspecific binding, as determined in the presence of 10 μM unlabeled ligand, was subtracted from the total binding to yield specific binding. In a preliminary set of experiments (data not shown), we verified that the presence of proteins (i.e., bovine serum albumin or rat plasma) in the binding buffer did not significantly affect the affinity of telmisartan for the AT1 receptor as it does with some other AT1 receptor antagonist (Maillard et al., 2001).

Kinetic Analysis of Binding

In association experiments, the amount of specific binding of 125I-AngII (10 μM) or the amount of specific cell-surface binding of [3H]telmisartan (5 nM) or [3H]candesartan (5 nM) to the living RVSMC (200,000 cells/well) were determined after various time intervals between 0.5 and 240 min. In dissociation experiments, [3H]telmisartan (10 nM) was incubated to steady state with membranes for 60 min. An excess of unlabeled AngII or cold telmisartan to the incubation mixture. Specific binding was defined as total binding minus nonspecific binding.

Data Analysis

Characterization of binding saturation curves (i.e., Scatchard analysis) and assessment of the number of AT1 receptors (Bmax) and the dissociation constant (Kd) of antagonist, were obtained using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The inhibition constant (K) values were calculated from the respective IC50 values using the Cheng-Prisoff equation: K = IC50/(1 + [L]/Kd), where [L] is the concentration of the radioligand and Kd is the dissociation constant obtained from Scatchard analysis and kinetic data. The IC50 values were determined by nonlinear least squares fitting of the inhibition curves with a sigmoid-Boltzmann equation (GraphPad Prism software). The dissociation rate constants (K-1) were calculated from the first-order plot of ln (Bt/Bm) versus time, where Bm and Bt are the amount of specific binding at equilibrium and time t. The half-life of the ligand receptor-complex (t1/2) was calculated using t1/2 = (ln 2/K-1). The observed rate constant (Kobs) was determined from the pseudo-first-order plot of ln (Bt/Bm) versus time. The association rate constant (K1)
was derived from $K_i = (K_{obs} - K_1)/(L)$. $K_d$ values from kinetic data were given as the ratio $K_1/K_d$.

**In Vivo Studies**

Experiments were performed on male, normotensive Wistar rats weighting 200 to 250 g obtained from Ills-Credo (L’Arbresle, France). These experiments were approved by an institutional committee for the humane use of animals. Animals were studied on a standard food (4 mg of Na+/g of pellet) ordered from Usine d’Alimentation Rationnelle (UAR; Epinay-sur-Orge, France) and tap water. All experiments were carried out in conscious semirestrained rats. Solutions for i.v. infusion were prepared by dissolving the drugs in a glucose-saline aqueous 0.2 M NaOH solution (pH 9.7 adjusted with HCl).

The day before the experiments, an arterial (polyethylene PP-10 in PP-50) and a venous (polyethylene PP-50) catheter were inserted into the right femoral artery and vein under halothane anesthesia. The catheters were filled with heparinized saline, tunneled subcutaneously, exteriorized, sealed, and emerged at the neck. Rats were allowed to recover from anesthesia overnight, and on the next day, they were placed in a Plexiglas tube to partially restrain their movements. Thirty minutes later, the arterial line was connected to a pressure transducer to measure mean arterial pressure and heart rate continuously using a computerized data-acquisition system. The venous catheter was used to infuse the drugs or to inject AngII. Blood samples were obtained through the arterial catheter at each time-points. They were drawn into a lithium-heparin Multivette 600-LH (Starstedt, Nürnberg, Germany). An equal volume of prewarmed gluco-saline solution was injected to compensate fluid loss. The presor responses to repeated infusion of exogenous AngII (100 ng/kg body weight) were measured before, 5 and 30 min, and 2, 4, 8, and 24 h after i.v. administration of either telmisartan (0.1, 0.3, or 1.0 mg/kg), irbesartan (0.3, 1.0, 3.0, or 10.0 mg/kg), candesartan (0.3 or 1.0 mg/kg), or the vehicle only (placebo group). AT1 blockade effect was assessed by the percentage of inhibition of the blood pressure increase to AngII.

**In Vitro-ex Vivo Assessment of Angiotensin II Receptor Blockade.** The degree of AT1 receptor blockade induced in rats treated by telmisartan was also measured ex vivo using the standardized radio-receptor assay described previously (Maillard et al., 1998, 2000a, 2002). To compare the activity of different doses in different rats, the results were normalized and expressed as (Bmax − Bp)/(Bmax − Bn), where Bn is the residual activity in the presence of the plasma X, Bp the nonspecific binding, and Bmax is the residual activity in the presence of the reference plasma. The antagonistic effect was evaluated using the ratio between the residual $^{125}$I-AngII bound to AT1 in presence of a plasma collected after drug intake and the amount of $^{125}$I-AngII bound to AT1 in presence of the plasma of the same rat, but collected before infusion of the drug.

**Plasma Telmisartan Concentration.** Plasma for pharmacokinetic measurements was drawn at each time point. The plasma telmisartan concentrations were determined by a validated competitive enzyme-linked immunosorbent assay using polyclonal rabbit anti-telmisartan antibodies, which were modified with biotin. Biotinylated antibodies were immobilized on avidin-coated microtiter plates. Free telmisartan in the sample competed with a fixed amount of added horseradish peroxidase conjugates of telmisartan for antigen binding sites on the plate surface. Bound enzyme conjugate was detected photometrically after incubation of the plate wells with a chromogenic substrate. This assay enabled the accurate and precise measurement of telmisartan in the range of 0.3 to 1000 ng/ml. Samples were diluted 10-fold with assay buffer before analysis. The assay calibration range was 0.03 to 100 ng/ml.

**Statistics**

Results are presented as the means ± S.E.M. unless stated otherwise. Data reported from in vitro assays were representative of several (2–5) separate experiments performed in duplicate or triplicate. The dose- and time-dependent effects of i.v. administration of drugs on the i.v. AngII-induced pressor response and the in vitro AT1 receptor blockade were calculated by repeated measure analysis of variance. Comparisons between the vehicle- and the drug-treated groups for each time point were also performed by analysis of variance. A $P$ value of $<0.05$ was considered statistically significant. Plots of the concentration-time curve were generated, and the elimination rate constant ($\lambda_e$) was estimated by the linear regression of at least three points that were in the terminal phase. The $t_{1/2}$ was then calculated as $(\ln 2)/\lambda_e$. The relationship between plasma drug concentrations and the percentage of inhibition of the pressor response to AngII were modeled using the Hill sigmoid $E_{max}$ equation (Hill, 1910). The same modelization was also performed with in vitro data.

**Results**

**Radioligand Binding Studies.** In intact RVSMC cells and in membrane preparations, telmisartan inhibits the binding of $^{125}$I-AngII to AT1 receptors in a concentration-dependent manner, with an IC$_{50}$ of 9.2 ± 0.8 nM. In the same experimental conditions, angiotensin II displaces $^{125}$I-AngII with an IC$_{50}$ value of 2.9 ± 0.5 nM. The specific binding of $[^{3}H]$telmisartan to SMC membranes is displaced by unlabeled telmisartan with an IC$_{50}$ of 7.7 ± 1.8 nM and by cold AngII with an IC$_{50}$ of 32.7 ± 5.7 nM. As shown in Fig. 1, the specific binding of telmisartan to RVSMC (cells or membranes) is saturable. The Scatchard analysis of the saturation binding data yields a linear plot, confirming that only a single population of binding sites is present on the surface of the vascular smooth muscle cells. A dissociation constant ($K_d$) for telmisartan of about 1.7 ± 0.3 nM is calculated with these experiments, and the $B_{max}$ is 0.15 pmol/mg of protein ($n = 3$).

Fig. 2 shows the time course of association and dissociation of $[^{3}H]$telmisartan binding to intact RVSMC. The specific cell surface binding of 5 nM labeled-telmisartan increased quickly and reached equilibrium after approximately 1 h. After data transformation (Fig. 2a), the observed association rate constant was determined ($K_{obs}$), and the association rate constant ($K_1$) was calculated at 0.0059 (± 0.0004) min$^{-1}$ nM$^{-1}$. Once the specific binding has reached equilibrium, dissociation was induced by washing the cells and adding fresh medium. The time course of the dissociation was then estimated by the release of radioactivity from the $[^{3}H]$telmisartan-receptor complex. The dissociation rate constant ($K_d$) calculated after linearization of these data was shown to be 0.0092 (± 0.0003) min$^{-1}$ (Fig. 2b), resulting in an initial
This confirms that candesartan reassociates with the receptor once it is dissociated.

**In Vivo Studies.** Figure 3 shows the effect of increasing doses of telmisartan on the blood pressure response to exogenous angiotensin II (upper panel) and the degree of angiotensin II receptor blockade assessed ex vivo using the radioreceptor assay (lower panel). The three single i.v. doses of telmisartan (0.1, 0.3, and 1.0 mg/kg) induced a dose-dependent inhibition of the pressor response to AngII. The blockade was long-lasting and remained significant more than 24 h at doses >0.1 mg/kg. With the 1 mg/kg dose of telmisartan, AngII receptors were blocked by 95 ± 2% (mean ± S.E.M.) at peak and by 78 ± 4% at 24 h. The ex vivo assessment of AT1 receptor blockade showed similar results (Fig. 3, lower panel). As reported previously with other compounds (Mazzolai et al., 1999; Maillard et al., 2000a,b), a close correlation was found between the two methods used to measure AT1 receptor blockade (r = 0.945; p < 0.0001). Figure 4 shows the relationships between plasma levels of telmisartan and pharmacodynamic activity (here, in vivo blockade of AngII pressure effect) calculated using an E_{max} model fitting with the Hill sigmoid curve. Notably, the same E_{max} and EC_{50} values were obtained regardless of the method of AT1 blockade assessment used.

Finally, Fig. 5 presents the results of the in vivo experiments comparing telmisartan, irbesartan, and candesartan. All three drugs produced a dose-dependent AngII receptor blockade, but irbesartan was found to be 10-fold less potent than telmisartan. At the dose of 1 mg/kg, candesartan was comparable to telmisartan.

**Discussion**

Taken together, the results of this study show that telmisartan, which has a high affinity for the angiotensin AT1 receptor, dissociates very slowly from the receptor once it is bound, and unlike candesartan, it does not reassociate with the receptor after dissociation. This slow dissociation may account for the insurmountable profile of the drug. When compared with two other long-acting angiotensin II receptor antagonists in the rat, telmisartan produces a comparable degree of angiotensin II receptor blockade and duration of

### Table 1

<table>
<thead>
<tr>
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<th>Association</th>
<th>Dissociation</th>
<th>K_{D}</th>
<th>K_{1}</th>
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<tbody>
<tr>
<td></td>
<td>K_{1}</td>
<td>t_{1/2}</td>
<td></td>
<td>K_{-1}</td>
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<tr>
<td>Telmisartan</td>
<td>0.0059 (±0.004)</td>
<td>75.3 (±2.5)</td>
<td>1.7 (±1.2)</td>
<td></td>
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<tr>
<td>Angiotensin II</td>
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<td>14.5 (±0.4)</td>
<td>0.7 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>Candesartan</td>
<td>0.0033 (±0.0010)</td>
<td>112.3 (±20.3)</td>
<td>1.9 (±1.0)</td>
<td></td>
</tr>
</tbody>
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Addition of fresh medium.
Addition of 10 μM telmisartan.
Addition of 10 μM AngII.
Addition of 10 μM losartan.
Addition of 10 μM candesartan.
blockade to irbesartan and candesartan, but telmisartan is 10-fold more potent than irbesartan.

In functional in vitro studies using rabbit aortic preparations, telmisartan has been shown to produce a rightward shift of the AngII concentration-contractile response curve together with a decrease in the maximal response of 40 to 50% (Wienen et al., 1993). One of the goals of the present study was to investigate the molecular basis of this peculiar behavior, called insurmountable antagonism. As mentioned earlier, several potential mechanisms have been proposed to explain the insurmountable profile of angiotensin II receptor antagonists (Vanderheyden et al., 2000b). In accordance with the recent work of Fierens and colleagues (1999b) and Vanderheyden et al. (2000a) who investigated other angiotensin II receptor antagonists, our data suggest that a slow dissociation from the receptor resulting in an increased longevity of the antagonist-receptor complex can represent one of the leading mechanisms for the insurmountable characteristic of telmisartan. Indeed, in our in vitro system, we found that the specific binding of [3H]telmisartan to angiotensin II AT1 receptors is rapid, time-dependent, reversible, and saturable. The equilibrium is reached within 1 h, and the dissociation of telmisartan from AT1 receptors is rapid, with an initial dissociation half-time of about 75 min compared with 14 min for AngII. In the same conditions, [3H]candesartan also dissociated slowly from AngII binding sites (1/2 of [3H]candesartan = 112 min). However, the dissociation rate of telmisartan is not affected by the presence of other competitors at the receptor level. This suggests that telmisartan does not reassociate once it dissociates from the receptor in contrast to candesartan (Fierens et al., 1999a,b; Vanderheyden et al., 2000a), which is also an insurmountable drug (Ojima et al., 1997; Morsing et al., 1999).

During angiotensin II receptor blockade in vivo, circulating angiotensin II levels increase (Christen et al., 1991). These increased levels of angiotensin II could potentially compete with the binding of the antagonist to the AT1 receptor and hence modulate the duration of the receptor blockade. Whether this does occur in vivo is not clear. In the present experiments, we have not only observed that AngII dissociates 5 times more rapidly from the AT1 receptors than telmisartan but also that AngII demonstrates lower affinity compared with telmisartan when competing with the binding of [3H]telmisartan to the AT1 receptor. In fact, our data demonstrate that the relative inhibitory potencies of telmisartan and AngII for each radioligand are different and that
the binding of radiolabeled substances is inhibited most potently by the respective unlabeled ligand. The reason for this difference is not entirely clear, but one hypothesis may be that the binding sites for AngII and nonpeptide antagonists do not overlap totally, as has been claimed for candesartan (Ojima et al., 1997) and other antagonists (Schantz et al., 1994), or that a possible allosteric modification (or modulation) of the AT1 receptors occurs in the presence of telmisartan. Thus, the insurmountable behavior of telmisartan might be explained in part by its slow dissociation from the receptors and also by the fact that angiotensin II is not the best competitor for telmisartan at the receptor level. Although the mode of AT1 receptor antagonism probably does not play a role in defining the antihypertensive effect of the antagonist (Timmermans, 1999), it is likely that a slow off-rate from the AT1 receptor may extend the time of occupancy of the receptor protein and lengthen the duration of antagonism.

The second objective of this study was to compare in vivo in rats the time profile of the angiotensin II receptor blockade induced by various doses of three long-acting angiotensin II receptor antagonists (i.e., irbesartan, candesartan, and telmisartan). AT1 receptor blockade was investigated using two different methods of investigation (i.e., by the repeated injections of exogenous AngII and monitoring of blood pressure increase and by the quantification of the degree of AngII receptor blockade in these animals using an ex vivo/in vitro radio-receptor assay, as described previously). As expected, all three antagonists blunted the pressor response to exogenous AngII dose dependently. The blockade was long lasting, and a significant residual blockade was found at 24 h. The results were similar regardless of the method used to evaluate the blockade, confirming the long duration of action of these compounds. The direct comparisons show that in the rat, telmisartan and candesartan have a rather similar profile, although the residual blockade at 24 h tended to be greater with telmisartan than with candesartan. The difference observed 24 h after dosing may be attributed to the pharmacokinetic profile of telmisartan, which has a particularly long elimination half-life. Indeed, the determination of the plasma concentrations of telmisartan during all these experiments allowed us to calculate the rate of elimination of this drug in rats. An elimination half-life ranging from 22 to 30 h was estimated for telmisartan in our experiments. By comparison, candesartan has an elimination half-life in rats of about 4 to 7 h (Kondo et al., 2002), whereas irbesartan is eliminated with an half-life of ca. 12 h (Davi et al., 2000). More impressively, telmisartan is found to be 10-fold more potent than irbesartan in the rat. The large difference in potency observed between telmisartan and irbesartan is not entirely explained by our data. In contrast to telmisartan, irbesartan has a very large volume of distribution, which may account for the difference. This factor may be particularly relevant since a single intravenous dose was administered to our animals.

In conclusion, this study confirms the long lasting action of the angiotensin II receptor antagonist telmisartan. It provides also further insights on the mechanisms leading to the insurmountable profile of the drug (i.e., the slow dissociation of the antagonist from the AT1 receptor and the fact that angiotensin II is not the best competitor for telmisartan at the receptor level).

The comparison of the activity of telmisartan with two other long-lasting angiotensin II receptor antagonists indicates that in rats telmisartan is as potent as candesartan but 10-fold more potent than irbesartan. Yet, we have to bear in mind that these observations are restricted to rat and that extrapolation about the potencies of these drugs in human is not straightforward. In addition, the parameters measured in this study only reflect the renin-angiotensin system blockade and not the antihypertensive effect of the drugs.

References


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