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Assessment of Angiotensin II Receptor Blockade in Humans Using a Standardized Angiotensin II Receptor-Binding Assay

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An in vitro angiotensin II (AngII) receptor-binding assay was developed to monitor the degree of receptor blockade in standardized conditions. This in vitro method was validated by comparing its results with those obtained in vivo with the injection of exogenous AngII and the measurement of the AngII-induced changes in systolic blood pressure. For this purpose, 12 normotensive subjects were enrolled in a double-blind, four-way cross-over study comparing the AngII receptor blockade induced by a single oral dose of losartan (50 mg), valsartan (80 mg), irbesartan (150 mg), and placebo. A significant linear relationship between the two methods was found ($r = 0.723$, $n = 191$, $P < .001$). However, there exists a wide scatter of the in vivo data in the absence of active AngII receptor blockade. Thus, the relationship between the two methods is markedly improved ($r = 0.87$, $n = 47$, $P < .001$) when only measurements done 4 h after administration of the drugs are considered (maximal antagonist activity observed in vivo) suggesting that the two methods are equally effective in assessing the degree of AT-1 receptor blockade, but with a greatly reduced variability in the in vitro assay. In addition, the pharmacokinetic/pharmacodynamic analysis performed with the three antagonists suggest that the AT-1 receptor-binding assay works as a bioassay that integrates the antagonistic property of all active drug components of the plasma. This standardized in vitro-binding assay represents a simple, reproducible, and precise tool to characterize the pharmacodynamic profile of AngII receptor antagonists in humans.

KEY WORDS: **Angiotensin II, receptor, antagonists, human, in vitro assay.**

Blockade of the renin-angiotensin system cascade with angiotensin converting enzyme (ACE) inhibitors is an effective therapeutic approach to the treatment of hypertension and congestive heart failure.[1] More recently, angiotensin II (AngII) receptor antagonists, which block the binding of Ang II to its AT-1-receptor, have become an interesting alternative to the use of ACE inhibitors.[2] Since the original characterization of losartan, the first nonpeptide orally active AT-1 receptor antagonist, several new compounds have been developed that demonstrate a high affinity and selectivity for the AT-1 receptor subtype.[3]

In the 1970s and 1980s, the first evidence that ACE inhibitors block the renin-angiotensin system in humans were obtained by demonstrating that these compounds were able to blunt the blood pressure response to exogenous AngI but not to AngII.[4,5] A

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similar approach has been used recently for the early clinical investigation of AngII antagonists. Thus, the pharmacodynamics (PD) of several AT-1 antagonists have been characterized assessing their ability to block the blood pressure response to exogenous AngII.[6–10] This approach has enabled to gain valuable information on the minimal effective dose of each compound and on the time profile of the drug-induced receptor blockade.

The administration of exogenous AngII remains a rather invasive and complex way to evaluate the degree of AngII receptor blockade. Therefore, this approach is never used in hypertensive patients. Determination of the reactive increase in plasma renin activity or plasma AngII levels could represent an alternative way to monitor indirectly AngII receptor blockade after administration of an antagonist.[6] However, these parameters are characterized by their individual variability due mainly to the heterogeneity of sodium intake and renal sodium handling by hypertensive patients.

In the present article, we describe an in vitro AngII receptor-binding assay that enables to monitor the degree of receptor blockade in standardized conditions. When compared to the in vivo method using the injection of exogenous AngII, the in vitro receptor-binding assay produces similar results but with a greatly reduced variability. This standardized assessment of antagonistic effect of drug active components in human plasma represents a simple and precise new tool to characterize the PD profile of AngII receptor antagonists in normotensive subjects as well as in hypertensive patients.

METHODS AND SUBJECTS

In Vitro Assessment of AngII Receptor-Binding Assay The receptor-binding assay is conducted on rat smooth muscle cell membranes expressing solely the angiotensin AT-1 receptor subtype.[11] To obtain cell membranes, rat aortic smooth muscle cells (SMC) are cultured in Dulbecco's modified eagle medium (Gibco BRL, Life Technologies, Paisley, UK) with 15% fetal calf serum (Seromed SO115, Fakola AG, Basle, Switzerland). When achieving about 90% confluence, the cells are scratched with a rubber policeman, recovered, and homogenized in an ice-cold buffer containing 0.25 mol/L sucrose, 5 mmol/L Tris/HCl, 5 mmol/L EDTA, pH 7.4, and a protease inhibitor cocktail (Complete, Boehringer Mannheim, Germany). The suspension is centrifuged at 50,000 *g* for 30 min at 4°C. The pellet is resuspended in a binding buffer containing 50 mmol/L Tris/HCl and 5 mmol/L EDTA at pH 7.4 and centrifuged a second time as described above. The membrane pellet is finally resuspended in the binding buffer at 1 mg of protein/mL, aliquoted, quick frozen in liquid N_2, and stored at −70°C. Cells are used after 5 to 17 passages. The protein content of the pellet is determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

The receptor binding is performed at 37°C for 1 h with 100 μg membrane proteins in 375 μL binding buffer in the presence of 5 fmol of labeled AngII (angiotensin II (5-L-isoleucine) tyrosyl-^{125}I-monoiodinated; DuPont, Boston, MA) and 25 μL of human plasma. The blanks and the standard curve are performed using 25 μL of a reference plasma (RP). In the present experiments, the RP consists of a pool of different plasmas obtained from healthy untreated volunteers. This plasma is stored at −70°C and thawed before use in the same conditions as the tested plasma. Nonspecific binding (B_0) is estimated by adding 10 μmol/L of unlabeled human AngII (Peninsula, Belmont, CA) to the incubation mixture. Separation of bound labeled AngII is achieved by centrifugation and residual radioactivity (B) determined by gamma counting. To compare the activity of different antagonists, the results are normalized and expressed as $(B - B_0)/(B_{RP} - B_0)$ where B_{RP} is the residual activity in the presence of the RP. Within each assay, separate competition-binding curves are performed with cold AngII and with the AT-1 receptor antagonist losartan to assess the reproducibility of the method and the quality of the membranes.

The precision of the method has been assessed by repeated measurements of plasma samples from several subjects in one assay using the same batch of SMC membrane preparation (within-assay precision) or in several assays with different batches of SMC membrane preparations and of labeled AngII (between-assay precision). The reproducibility (R) of the method has been estimated by analyzing the results obtained with the plasmas of 12 volunteers collected at times 0, 4, 24, and 30 h after administration of a placebo. These plasmas contained no AngII receptor antagonist susceptible to displace binding of ^{125}I–AngII. The reproducibility is calculated as a mean coefficient of variation using the following formula:

$$R = \sqrt[2]{\sum_{i=1}^{n} \frac{(CV_i)^2}{n}},$$

where CV is the coefficient of variation in subject *i* for three time different measures and n the number of volunteers. The same formula has been used to calculate the reproducibility of the in vivo method.

Comparison of the In Vitro With the In Vivo Assessment of AngII Receptor Blockade To validate the use of the in vitro AngII receptor-binding assay, the results obtained in vitro were compared to those gathered in vivo with the injection of exogenous AngII and the measurement of the AngII-induced changes in

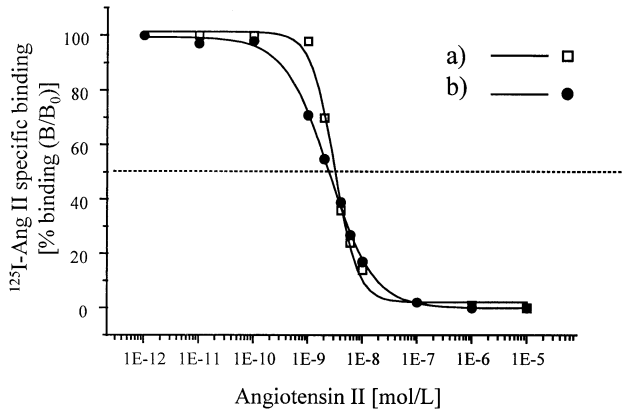

FIGURE 1. *Specific ^{125}I–AngII binding in rat aortic SMC membrane preparation in presence of unlabeled AngII with either 25 μL of BSA 7% (***a***) or 25 μL of human plasma (***b***).*

systolic and diastolic blood pressures. To this purpose, blood was collected in 12 normotensive subjects enrolled in a double-blind, placebo-controlled, four-way cross-over study dedicated to the comparison of the AngII receptor blockade induced by a single oral dose of losartan (50 mg), valsartan (80 mg), and irbesartan (150 mg).[12] Blood was collected before the administration of each compound and 4, 24, and 30 h after drug intake into 2.7 mL of S-Monovette KE (Sarstedt, Nümbrecht, Germany) containing 1.6 mg of EDTA/mL. Blood was taken systematically before the administration of exogenous AngII.

The in vivo assessment of AngII receptor blockade was performed as reported previously.[6] Briefly, a predetermined dose of exogenous AngII increasing systolic blood pressure by 25 to 40 mm Hg is administered intravenously before and 4, 24, and 30 h after drug intake. Blood pressure is measured and recorded continuously at the finger by photoplethysmography (Finapres, Ohmeda, Englewood, CO) shortly before and during several minutes after each injection of AngII and the peak blood pressure changes are calculated.[13]

The in vivo and in vitro methods were compared by analyzing the relationship between the in vivo assessment of AngII receptor blockade expressed as the percentage of the baseline systolic blood pressure response to AngII and the percentage displacement of ^{125}I–AngII in the in vitro assay.

Relationship Between Plasma Drug Levels and AngII Receptor Blockade Plasma drug levels were measured on time 0, 4, 24, and 30 h. Hence, the rela-

TABLE 1. PRECISION OF THE IN VITRO ASSAY

Samples	Percent ^{125}I-Ang II-Specific Binding		
	Mean	SD	Coefficient of Variation (%)
Within-assay precision (n = 10)			
Plasma 1	90	4.5	5.0
Plasma 2	68	3.2	4.3
Plasma 3	2.0	0.2	10.3
Between-assay precision (n = 7)			
Plasma 4	104.0	8.6	8.3
Plasma 5	55.0	1.7	3.0
Plasma 6	4.7	1.6	34.9

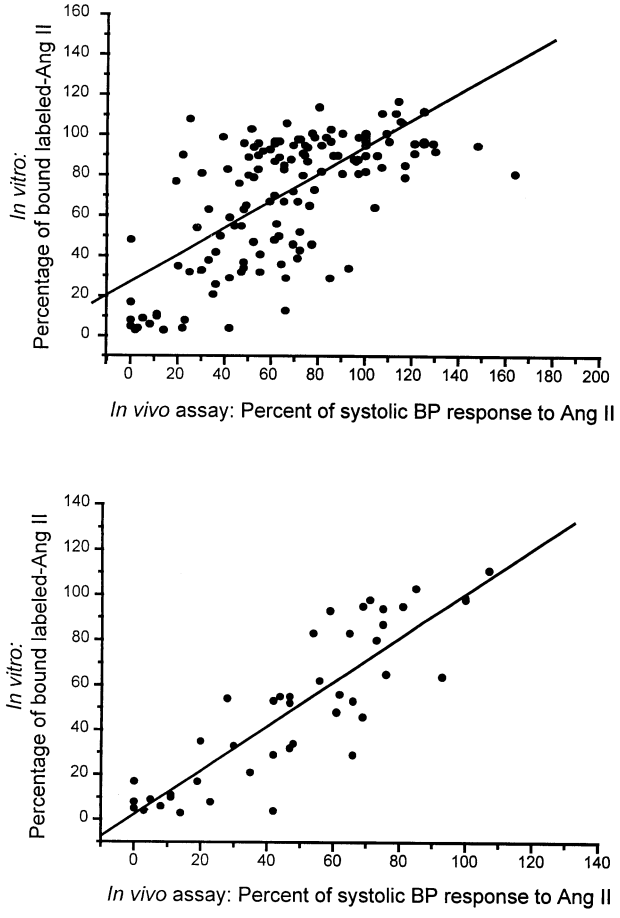

FIGURE 2. *Comparison of the in vivo and in vitro methods by analyzing the relationship between the in vivo assessment of AngII receptor blockade expressed as the percentage of the baseline systolic BP response to AngII and the percentage of labeled AngII in the in vitro assay. (***A***) All data (ie, AngII antagonistic activities measured in all volunteers, before and 4, 24, and 30 h after drug intake, within the four treatments). Linear regression: Y = 0.670 (±0.046) × +27.33 (±3.75) (r = 0.723; n = 191; P < .0001). (***B***) Data acquired during AngII receptor blockade, 4 h after drug intake. Linear regression: Y = 0.983 (±0.081) × +2.28 (±4.59) (r = 0.876; n = 47; P < .0001).*

TABLE 2. RELATIONSHIPS BETWEEN PLASMA DRUG LEVELS AND INHIBITORY EFFECT

Model	Valsartan		Irbesartan	
	In Vivo Assay	In Vitro Assay	In Vivo Assay	In Vitro Assay
log/lin	A = −3.70	A = −37.83	A = 9.75	A = −4.19
	B = 21.86	B = 32.96	B = 25.60	B = 35.56
E = A + B*Log (C)	r = 0.475	r = 0.922	r = 0.649	r = 0.951
Hill sigmoid	E_{max} = 78%	E_{max} = 75%	E_{max} = 83%	E_{max} = 100%
	EC_{50} = 63.9 ng/ml	EC_{50} = 193.4 ng/ml	EC_{50} = 24.3 ng/ml	EC_{50} = 34.7 ng/ml
$E = \frac{C * E_{max}}{C + EC_{50}}$	SS = 1.6717	SS = 0.6784	SS = 1.1552	SS = 0.1239

tionship between the pharmacokinetic (PK) and the PD effects of the AngII antagonists could be examined. To this purpose, individual values of the plasma drug concentrations were plotted against the individual PD variables and analyzed in a descriptive way. The PK/PD relationships were assessed with the percentage inhibition of the effects of the predefined dose of AngII (in the in vivo approach) and of the binding of labeled-AngII in the in vitro assay. Two models were used to describe the PK/PD: a log-linear model, where the effect is related to the log of the plasma concentration and an E_{max} model fitting with the Hill sigmoid curve given as follows[14]: $E = (C \times E_{max})/(C + EC_{50})$, where C is the drug level in the plasma, EC_{50} is the drug concentration that produces half the maximal effect, and E_{max} is theoretic maximal effect observable.

The PK/PD relationship of losartan was more complex to assess as this AngII antagonist is metabolized in the liver to EXP3174, a metabolite that represents the major active component of losartan potassium.[7] Therefore, the PK/PD analysis was performed with plasma EXP3174 alone and also considering both plasma losartan and EXP3174 levels. To take into account the relative affinity of each active components to the AT-1 receptor, the respective concentration of losartan and EXP-3174 were expressed as concentration equivalents and were presented as multiples of the concentration at which 50% of receptors were occupied by the antagonists ($n \times Ki$).[15] It should be mentioned that in contrast to the concentration, the concentration equivalent is given without dimension.

Statistical Analysis The correlation coefficients were calculated by the least square method. The in vitro/in vivo comparison was done by a one-way ANOVA. A one population *t* test was also performed on the mean difference (in vitro − in vivo) to analyze the difference from 0. All values are expressed as mean ± SEM unless otherwise indicated. A P value $< .05$ is considered as significant. Hill sigmoid curves fitting are performed with the solver tool of Microsoft Excel version 7.0 (Microsoft Corporation, Redmond, WA). E_{max} and EC_{50} are estimated by an iterating procedure minimizing the sum of squared differences between calculated and observed effect values.

RESULTS

Characteristics of the In Vitro Receptor-Binding Assay A typical ^{125}I–AngII displacement curve by unlabeled AngII in a rat aortic SMC preparation is shown in Figure 1. In the presence of 25 μL of a 7% bovine serum albumin (BSA) solution, the half-maximal inhibition concentration (IC_{50}) is 3.1 ± 0.2 nmol/L. The substitution of BSA by 25 μL of the human RP containing no drug does not significantly modify the curve (IC_{50} shifted to 2.5 ± 0.1 nmol/L) (Figure 1). In the same assay, the AT-1 antagonist DUP753 (losartan) inhibits the binding of radiolabeled AngII with an IC_{50} of 10.7 ± 2.2 nmol/L. To determine the effect of the protein content on the displacement of ^{125}I–AngII by an AngII antagonist, displacement curves were carried out with losartan in assays containing increasing concentrations of albumin in the binding buffer. With protein concentrations ranging between 45 and 90 g/L, no significant effect of the protein content on the displacement curve was found.

Table 1 summarizes the results obtained by 10 measurements of three different plasma samples covering a large range of antagonistic activity (within-assay precision). The coefficient of variation varied between 4.3% and 10.3% depending on the degree of antagonistic activity. For active plasmas with a low percentage of ^{125}I–AngII-specific binding, the variation is higher but with a very small imprecision on the measurement of bound labeled AngII. Between-assay precision evaluated on three samples measured repeatedly in seven consecutive assays ranges between 3.0% and 8.3% for plasma showing either no or little antagonistic activity. The CV increased to 35% with samples exhibiting an almost complete antagonistic activity.

Comparison With the in Vitro Assessment of AngII Receptor Blockade With the In Vivo Approach Figure 2A shows the relationship existing between the

TABLE 2. CONTINUED

Model	Losartan (EXP3174)		Losartan (EXP3174 + Losartan)	
	In Vivo Assay	In Vitro Assay	In Vivo Assay	In Vitro Assay
log/lin	A = 9.17 B = 13.8	A = −30.30 B = 42.02	A = 5.98 B = 13.86	A = −39.03 B = 41.51
E = A + B*Log (C)	r = 0.313	r = 0.770	r = 0.321	r = 0.872
Hill sigmoid $E = \frac{C * E_{max}}{C + EC_{50}}$	E_{max} = 41% EC_{50} = 8.9 ng/ml SS = 1.2222	E_{max} = 100% EC_{50} = 75.5 ng/ml SS = 0.3051	E_{max} = 40% EC_{50} = 12.7* SS = 1.2675	E_{max} = 99% EC_{50} = 134.6* SS = 0.2955

*E, Effect expressed in percentage inhibition (resp. AngII response in vivo and $_{125}$I-AngII binding in vitro); C, plasma concentration of drug [ng/mL] or * expressed as concentration equivalents (n × Ki).*

In log/lin model, A and B, respectively, represent the intercept and the slope of the constructed regression line, and r is the correlation coefficient of this line.

In the Hill sigmoid model, E_{max} represents the theoretical maximal inhibition; EC_{50} = drug concentration that produces half the maximal effect; SS = sum of squares of the difference between $E_{measured}$ and $E_{estimated}$ on the Hill sigmoid curve.

percent inhibition of systolic blood pressure in response to exogenous AngII measured in vivo and the percentage of bound radiolabeled AngII in vitro. A significant linear relationship between the two methods is found (r = 0.723, n = 191, $P < .001$). However, the figure demonstrates that there is a wide scatter of the in vivo data when no in vitro AngII receptor blockade is measured. Figure 2B shows the relationship between the two methods when only measurements done 4 h after administration of the AngII antagonists are considered (maximal antagonist activity observed in vivo). In this case, the relationship is markedly improved (r = 0.87, n = 47, $P < .001$) suggesting that as soon as some blockade of the AngII receptor occurs, the two methods are equally effective in assessing the degree of AT-1 receptor blockade. Noteworthy, the slope of the regression curve is close to 1 and the intercept is near 0, indicating that the curve almost fits with the identity line. Statistically, no significant difference between values measured with the two approaches was found.

Our results suggest, however, that the in vivo approach has a greater variability than the in vitro method. When the methods are compared 4, 24, and 30 h after the administration of a placebo, the variability of the blood pressure response to exogenous AngII is close to 40%, whereas the variation of the inhibition of ^{125}I–AngII binding is only 7% with the in vitro assay. In this study, the mean overall intraindividual CV for repeated measurements was, respectively, 25.4 ± 2.7% (n = 12; range, 8% to 40%) with the in vivo method and 5.2 ± 2.4% (range, 1% to 9 %) with the in vitro receptor-binding assay. The overall reproducibility of both methods was, respectively, 28.1% and 5.6% for the in vivo and the in vitro assessments of AngII receptor blockade.

The variability of the blood pressure response to exogenous AngII appears to be lower when several injections of the same dose of AngII are administered within a shorter time. Thus, when defining the dose of AngII increasing systolic blood pressure by 25 to 40 mm Hg, three consecutive injections of the same amount of angiotensin were administered to our subjects at 30-min intervals. Under these conditions, the mean intraindividual CV of the blood pressure response was 13.4 ± 1.1% (range, 2.1% to 43%) and the overall reproducibility of the in vivo method estimated at 13.9%.

Use of the In Vitro Receptor-Binding Assay in PK/PD Analysis Table 2 shows the PK/PD relationships of valsartan, irbesartan, and losartan in the 12 normotensive subjects. Plasma drug levels were correlated either with the percentage changes in blood pressure response to exogenous AngII or with the results of the in vitro receptor-binding assay in the two analytical models chosen for assessing PK/PD relationships of AngII antagonists. With valsartan and irbesartan, the PK/PD relationship was significantly improved by the use of the in vitro data with r values > 0.9 in the log/linear model (see Figure 3) and very low sum of squares values in the E_{max} model (see Table 2). Estimation of the PK/PD profile of losartan using plasma EXP3174 levels gave poor results with the in vivo data as no significant correlation was found with the log/linear or the E_{max} model. When analyzed based on the in vitro data, the PK/PD profiles of losartan were more consistent with the models used. The fitting with both models was still improved when the sum of all active drug components present in plasma was taken into account (ie, EXP3174 and plasma losartan levels, expressed as the sum of their

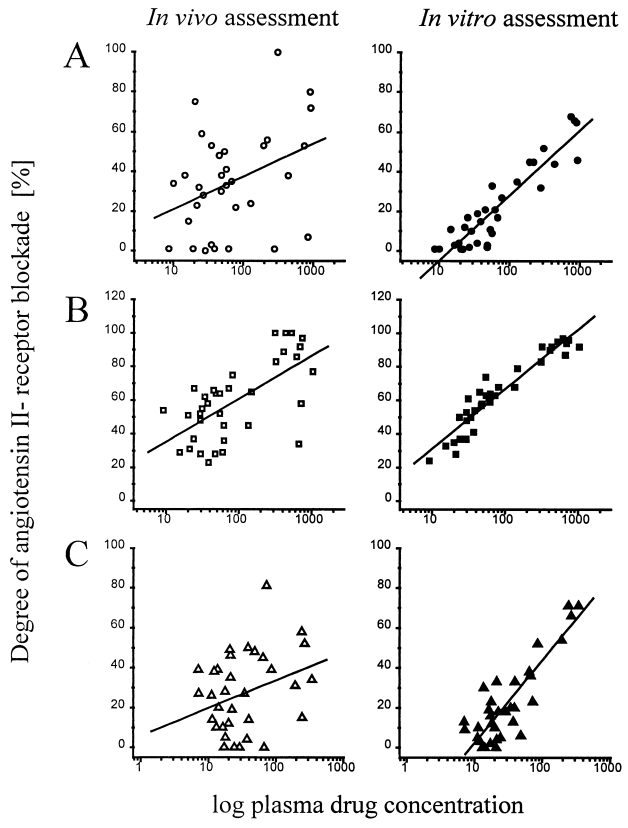

FIGURE 3. *Relationships between plasma drug levels and inhibitory effect. The effect (percentage of blockade of the pressor response to AngII in the in vivo approach [open symbols], and percentage of ^{125}I–Ang II displaced from receptor in the radioreceptor assay [solid symbols]) is related to the log of the plasma concentration of (***A***) valsartan (○/●), (***B***) irbesartan (□/■), or (***C***) losartan and EXP-3174 (△/▲). The drug concentrations are expressed in ng/mL for valsartan and irbesartan and in concentration equivalents (n × Ki) for the sum of losartan and EXP-3174 (see explanation in text).*

respective concentration equivalents). In these conditions, closer relationships were observed (with r value approaching 0.9 [$P < .001$] in the log/linear model and lower SS values in the E_{max} model).

DISCUSSION

This article presents an in vitro AngII receptor-binding assay that enables to assess the AT-1 receptor antagonistic effect of human plasma in well-standardized conditions. When compared with the in vivo approach that uses the injection of exogenous AngII to evaluate the degree of AngII receptor blockade, this assay produces comparable quantitative results in subjects receiving an AngII receptor antagonist but with a greatly reduced inter- and intraindividual variability. The PK/PD analysis performed with three antagonists suggests that the AT-1 receptor-binding assay works as a bioassay that integrates the antagonistic property of all active drug components of the plasma (ie, active drug and active metabolite). This in vitro-binding assay that avoids the use of exogenous AngII represents a simple, reproducible, and precise new tool to characterize the PD profile of AngII receptor antagonists in humans.

The in vitro receptor-binding assay offers several advantages over the past techniques used to evaluate a drug-induced blockade of AngII receptors. First, the assay is specific as it is performed on vascular smooth muscle cells that contain only AT-1 and no AT-2 receptors, as demonstrated previously.[11] Second, the use of a membrane preparation allows to prepare a large amount of material at one time and to keep the membranes frozen without damage. Thus, the same batch of membranes can be used throughout a study, thereby reducing, considerably, the interassay variability. A similar assay has been developed recently using rat lung tissue.[16] The advantage of rat SMCs is that they are now commercially available and easy to grow in culture. As shown in previous experiments and again in this study, the IC_{50} for cold AngII and losartan is in the low nanomolar range.[17,18] These values reflect the integrity of AT-1 receptors in the membrane preparation. Thus, these parameters can be used as internal quality controls.

The addition of 25 μL of human plasma to the binding buffer does not interfere with the characteristics of the ^{125}I–AngII displacement curve. Although human plasma contains AngII, its endogenous concentration stays at levels that do not interfere with the competition binding measured in the assay (between 2 fmol/mL before the administration of any drug and about 40 fmol/mL during maximal AngII receptor blockade). In addition, the presence of EDTA in plasma samples and in binding buffer prevents enzymatic production of AngII or degradation of drugs during the incubation process.[19] The small amount of plasma needed to perform the assay certainly represents another advantage of the method. Indeed, this enables to perform several measurements in the same individual and thereby to improve the PD characterization of an AngII receptor antagonist.

When compared with the results obtained in vivo with the administration of exogenous AngII, the data gathered with the in vitro assay appear to be quantitatively similar and no significant difference was found between the two methods. Yet, the reproducibility of the in vitro assay is markedly better at 5% to 6%. The greater variability of the in vivo approach is not entirely surprising. The blood pressure response to exogenous AngII is known to differ markedly between individuals and to vary during the day. Other factors, such as meals and the subject's emotional response, can contribute to the variability of the blood pressure response to exogenous AngII. Interestingly,

the results obtained with the injections of AngII correlate very well with the in vitro data when an AT-1 receptor antagonist is administered. This suggests that the reproducibility of the in vivo method is improved as soon as there is a partial or complete blockade of AT-1 receptors. The variability of the in vivo method is also reduced when several injections of AngII are performed within a short time. In this study, exogenous AngII was injected up to 30 h after drug intake. This may explain why the variability of the in vivo approach was higher. Of course, the main advantage of the in vitro receptor-binding assay is that an adequate evaluation of AngII receptor blockade can be obtained without the administration of AngII. This is of particular interest when conducting PD studies directly in hypertensive patients. In addition, the very low variability of the method should enable to obtain reliable results with only a small number of subjects.

Irbesartan and valsartan are two AngII receptor antagonists that have no active metabolite. When the degree of AngII receptor blockade measured with the in vitro assay after administration of these two agents was correlated with plasma drug levels in two different PK/PD models, excellent correlations were found suggesting that the in vitro assay works as a bioassay assessing the overall antagonistic capacity of the tested plasma. This is further supported by our data using losartan. Indeed, when the PK/PD modelization was performed with the plasma levels of the active metabolite EXP3174 only, poor relationships were obtained. However, significant correlations were observed when the effect was correlated with the plasma content of both active components of the drug (ie, losartan and its principal metabolite EXP3174). The PD parameters (E_{max} and EC_{50}) measured in our study were in good agreement with those already published in the literature where Müller et al[8] estimated the theoretic maximum effect for valsartan to be 74% with an EC_{50} of about 200 ng/mL or for losartan where E_{max} of 91% and EC50 of 35 ng/mL were calculated.[7] To our knowledge, no data were published yet for irbesartan.

Whether the assay reflects only the activity of the free components of plasma drug levels is difficult to ascertain from our studies. The results of the PK/PD analysis calculated with the in vitro data are very comparable to those published previously with different PD assessments of AngII receptor blockade.[20]

In conclusion, this study presents a new in vitro AT-1 receptor-binding assay that enables to evaluate AngII receptor blockade with a high specificity and a very low variability. By avoiding the use of exogenous AngII, this method provides the opportunity to obtain standardized PD data in healthy subjects as well as in hypertensive patients.

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