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A radioreceptor assay for the analysis of AT_1-receptor antagonists
Correlation with complementary LC data reveals a potential contribution of active metabolites

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Abstract

A reliable and sensitive radioreceptor assay based on rat lung homogenate as receptor preparation was developed to determine the angiotensin-II antagonistic profile of losartan and its main active metabolite EXP 3174 as well as its congeners exemplified by UP 269-6 and SL 91.0102-90 DL. This method proved to be precise with an intra- and interday variability of less than 10% and a limit of quantification ≤ 1 ng ml^{-1}. The analysis of the K_i values in protein-free Hepes-buffer versus blank human or rat plasma revealed the distinct high plasma–protein binding of EXP 3174 which consequently caused a dramatic drop of potency from 10–15-fold in the buffer to only about 2-fold in control plasma, when compared to the parent compound losartan and the two congeners investigated. Upon evaluation of clinical samples by both the reported radioreceptor assay (RRA) and the established high-performance liquid chromatography (HPLC), the correlation of the normalized data pairs (concentration equivalents) suggested the contribution of active metabolites to the angiotensin-II antagonistic effect of SL 91.0102-90 DL, but not to the effect of UP 269-6. In the context of an extended preclinical study in rats, the correlation of RRA with the respective HPLC concentration equivalents of losartan and its main active metabolite EXP 3174 confirmed previous findings that only losartan and EXP 3174 exert the angiotensin-II-AT_1 receptor blockade without the contribution of other metabolites (P.C. Wong, W.A. Price, A.T. Chiu et al., J. Pharmacol. Exp. Ther. 255 (1990) 211–217). Published by Elsevier Science B.V. All rights reserved.

Keywords: AT_1-receptor; Losartan; EXP 3174; UP 269-6; SL 91.0102-90 DL; Radioreceptor assay

Abbreviations: RP-HPLC, Reverse-phase high-performance liquid chromatography; RRA, Radioreceptor assay.

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1. Introduction

Angiotensin II (AII) represents a potent effector peptide of the renin–angiotensin–aldosterone system (RAAS) that exerts a wide variety of physiological actions on the cardiovascular, renal, endocrine, and central nervous systems [1–3]. The biological effects of AII are mediated through the binding of AII to specific cell surface receptors (in particular, receptors of the AT_1- or AT_2-subtype). AT_1-receptors have been shown to mediate many of the physiological effects commonly associated with an (over-) stimulated RAAS such as vasoconstriction and elevated blood pressure, aldosterone and noradrenaline release.

Since the discovery of nonpeptide angiotensin-II AT_1- and AT_2-antagonists [4–8], numerous tissues from rats, rabbits and humans, which often express more than one subtype, have been studied in respect to their angiotensin-II subtype profile [9–12]. Using rat lung that exclusively exhibits the AT_1-receptor subtype [13,14], we focused on the development of a universally applicable radioreceptor method for angiotensin-II antagonists in order to characterize the AT_1-affinity profiles of the prototype losartan as well as of selected congeners in that tissue.

In general, the knowledge of the inhibition constants K_i of antagonistic drugs enables the determination of their concentrations in unknown samples as concentration equivalents, i.e. multifolds of K_i. In contrast to the selective determination of different compounds in the micro- and nanomolar range by HPLC, the radioreceptor assay measures in picomole quantities, but does not distinguish between different active compounds. Consequently, the comparison of data resulting from HPLC measurements versus RRA measurements enables evaluation, whether the compounds analyzed by HPLC are the (only) active ones at the receptor site or whether active metabolites are formed and are therefore present in the sample. Such active metabolites, which exhibit receptor affinity, may contribute to the displacement of the radioligand measured in the radioreceptor assay.

In this context, the correlation of normalized concentration equivalents from both binding studies and liquid chromatography was anticipated to reveal a 1:1 relationship in the case where only the parent drug was active. In contrast, if the receptor blockade determined in the radioreceptor assay was achieved by the parent compound and/or active metabolites, the correlation with concentration equivalents of the parent drug only was assumed to result in a $1+n$:1 relationship with n describing the extent of efficacy of the active metabolites involved.

The aim of the present study was therefore to establish a radioreceptor assay for the in vitro assessment of the AT_1-affinity profile of selected angiotensin-II antagonists. Upon determination of preclinical and clinical samples using this radioreceptor assay and previously established HPLC methods, the correlation of the respective data pairs standardized as concentration equivalents is shown herein as a means to elucidate the potential contribution of active metabolites to the observed angiotensin-II antagonistic effect.

2. Experimental

2.1. Materials

Losartan and EXP 3174 were kindly provided by Merck Research Laboratories (Rahway, NJ), SL 91.0102-90 was from Synthelabo Recherche (Munich, Germany) and UP 269-6 was from UPSA (Paris, France). [^{125}J](Sar^1-Ile^8)-angiotensin II was purchased from Anawa (Wangen, Switzerland). Angiotensin II was obtained from Bachem Biochemica (Heidelberg, Germany). Extra pure Hepes (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), Tris (tris(hydroxymethyl) - amino - methane), Na_2-EDTA, $MgCl_2$ (hexahydrate) and NaCl were from Merck (Darmstadt). Bacitracin was purchased from Aldrich (Steinheim, Germany), bovine serum albumin from Serva (Heidelberg, Germany), and polyethylene imine (50% in purified water) from Sigma (Deisenhofen, Germany).

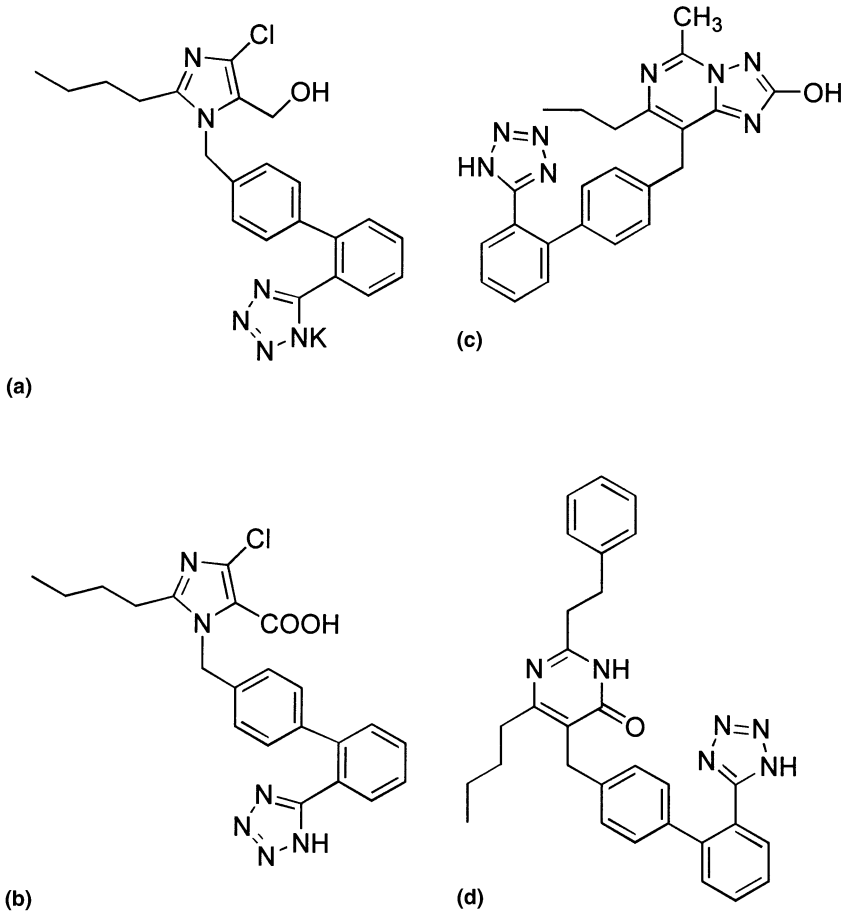

Fig. 1. (a) Losartan; (b) EXP 3174; (c) UP 269-6; (d) SL 91.0102-90 DL.

2.2. Instrumentation

Rat lung membranes were homogenized by using an Ultra-Turrax (T25, Janke and Kunkel, Staufen, Germany) and a Potter (B. Braun, Melsungen, Germany). Separation filtration was performed with a Brandel cell-harvester M-24R (Biomed, Gaithersburg, USA). A Wallac 1480, Wizard™ 3″ (Wallac, Turku, Finland) served for radioactivity measurements.

2.3. Buffers

Tris-buffer pH 7.2 was used during membrane preparation and consisted of 50 mM TRIS, 150 mM NaCl and 5 mM Na_2EDTA. Hepes-buffer was used as the assay buffer and consisted of 20 mM Hepes, 1 mM Na_2EDTA and 10 mM $MgCl_2$ (hexahydrate).

2.4. Biological samples

Three different sets of samples were investigated in this study. Samples were thawed only once to avoid changes in plasma–protein binding.

(a) The plasma samples containing losartan and its main active metabolite EXP 3174 (Fig. 1a, b) were obtained from an extended rat study on the disposition characteristics of losartan and EXP 3174 following single intraperitoneal administration of losartan (3 mg per kg body weight) to 48 rats ($n = 4$ for 12 time points) weighing 250 ±

Table 1
Dosing schedule for various doses of UP 269-6, placebo and enalapril 20 mg (randomized)

UP 269-6 (mg)	5	10	20	40	80	120	180	Placebo	Enalapril
Group 1	X		X		X			X	
Group 2		X		X				X	X
Group 3			X		X		X	X	
Group 4				X		X		X	X

20 g [15]. Plasma samples were withdrawn at appropriate time intervals up to 36 h post-dose and analyzed by HPLC and RRA.

(b) The samples containing UP 269-6 (Fig. 1c) were taken from a previously performed study in male volunteers comparing the antagonistic effect of various doses of UP 269-6 on the pressor action of exogenous angiotensin I to the ACE inhibitor enalapril [16]. In a double-blind and placebo-controlled study, four groups (16 volunteers in total) were randomized to receive either UP 269-6, placebo or enalapril (Table 1). Plasma samples for RRA and HPLC were obtained before drug administration and 1, 4, 10 and 24 h post dose.

(c) The samples containing SL 91.0102-90 DL (Fig. 1d) were taken from a study investigating the extent and duration of the depressor effects of SL 91.0102-90 DL on the exogenous administration of angiotensin II (Synthelabo Recherche, unpublished results). In a double-blind and placebo-controlled study, 15 male volunteers were randomized to receive either 25, 50 or 100 mg of SL 91.0102-90 DL or placebo. Plasma samples for RRA and HPLC were obtained before drug administration and 2, 4, 6, 10 and 24 h post dose.

The animal studies were carried out in accordance with 'guidelines for the use of laboratory animals' by the Biocenter of the University of Frankfurt/Main, Germany. Both clinical studies (Section 2.4b, Section 2.4c) were approved by the local ethical review committees.

2.5. Methods

2.5.1. Preparation of membrane fractions

Following a blow to the neck, lungs were dissected from male Wistar rats weighing 200–250 g each and immediately stored on ice. All future steps were performed at 4°C. The purified tissue was weighed, homogenized in Tris-buffer pH 7.2 with an Ultra-Turrax as well as a Potter and centrifuged at $1000 \times g$ for 10 min at 0°C. The supernatant was centrifuged at $20\,200 \times g$ for 20 min at 0°C and the resulting pellet was washed with Tris-buffer pH 7.2. The final pellet was resuspended by homogenization in Hepes-buffer pH 7.4, divided into aliquots and stored at -80°C at 1 mg ml^{-1} protein concentration (Bio-Rad protein assay) for use in binding assays.

For the binding assay, frozen membrane fractions were thawed, pooled and centrifuged at $20\,200 \times g$ for 20 min at 0°C. The pellet was resuspended by homogenization in Hepes-buffer pH 7.4 containing bacitracin (1 mM) and BSA (0.1%) to a final concentration of 1 mg protein per 1 ml buffer.

2.5.2. [^{125}J](Sar^1-Ile^8)-angiotensin II binding assay

For saturation as well as displacement studies, rat lung homogenate was incubated in either Hepes-buffer or blank human or rat plasma yielding 50 μg protein per well in a total assay volume of 300 μl. To ensure equilibrium conditions (see binding kinetics), the samples were maintained for 60 min at 25°C under continuous rotation. All experiments were carried out in duplicate, and the results were confirmed with $n = 3–5$ replicates over the following days.

2.5.3. Saturation experiments

As outlined above, lung homogenate was incubated with increasing (0.025–3.5 nM) concentrations of [^{125}J](Sar^1-Ile^8)-angiotensin II. Non-specific binding (approximately 15% of total

binding) was determined with 1 μM of unlabelled angiotensin II.

2.5.4. Displacement experiments

Lung homogenate was incubated with a fixed [^{125}J](Sar^1-Ile^8)-angiotensin II concentration of 0.06 nM and increasing concentrations of either losartan, EXP 3174, SL 91.0102-90 DL or UP 269-6.

In all studies, bound and free radioligands were separated by rapid filtration through Whatman GF/C glass fiber filters presoaked with an aqueous 0.1% solution of polyethylene imine, where the filters were rinsed twice with 5 ml ice-cold saline. Tissue-bound radioactivity was measured in a γ-counter at 75% efficiency.

2.5.5. HPLC analysis

(a) The concentration-time profiles of losartan and EXP 3174 in rat plasma were determined by solid-phase extraction using cyanopropyl-endcapped silica columns and RP-HPLC/UV-detection, as described previously [15].

(b) The UP 269-6 drug levels were assessed within UPSA laboratoires by chloroform liquid–liquid extraction and RP-HPLC/spectrofluorimetry, as described in [16,17].

(c) The SL 91.0102-90 DL drug levels were analyzed within Synthelabo Recherche by diethylether liquid–liquid extraction and RP-HPLC/spectrofluorimetry (Synthelabo Recherche, unpublished results).

2.6. Data analysis

K_D and B_{max} were evaluated by fitting the following equation with the saturation binding data using the nonlinear least-squares fitting computer program Sigma Plot™ for Windows, Version 2.01 (Jandel Scientific, San Rafael, CA):

$$RL^* = B_{max} \times L^*/(K_D + L^*) + nsb \times L^*$$

where RL* is the specifically bound (receptor-bound) radioligand; B_{max} is the maximal binding sites; L^* is the free radioligand concentration; K_D is the equilibrium dissociation constant, radioligand concentration at half-maximal receptor occupancy during equilibrium; and nsb is the non-specifically bound radioligand.

Saturation data were also transformed for graphical Scatchard and Hill analysis (Fig. 2). All values are given as mean $\pm$ SD for $n = 3–5$.

The K_i-values characterizing the AT_1-affinity profiles of the antagonists tested were calculated by extension of the above equation with the term I/K_i

$$RL^* = B_{max} \times L^*/[K_D(1 + I/K_i) + L^*]$$

where I is the concentration of the inhibitor (antagonist); and K_i is the equilibrium dissociation constant of the inhibitor.

Following evaluation of the biological samples (after administration of losartan, UP 269-6 or SL 91.0102-90 DL) by both radioligand binding studies and HPLC, the respective data pairs were transformed into multiples of K_i (n^*K_i) for comparison of the data as concentration equivalents:

nK_i(HPLC): P(g/l)/MW/K_i

nK_i(RRA): (P(M) + PAM(M))/K_i

where P is the parent drug (i.e. losartan, UP 269-6 or SL 91.0102-90 DL); PAM is the potentially active metabolite(s); MW is the molecular weight; and M is the molarity.

The concentration equivalents from RRA analysis which describe the total AT_1-receptor occupancy, i.e. by the antagonistic parent drug and/or its metabolites, were then correlated with the concentration equivalents from the HPLC analysis exclusively from the parent drug.

2.7. Radioreceptor assay validation

2.7.1. Intra- and interday variability

Six determinations of standards with a known receptor occupancy were performed on the same day (intraday) or on three consecutive days (interday). The percent deviation from the mean and SD were calculated for all samples and used as indicators for intra- or interday variability (Table 2). Intra- and interday variabilities were characterized by the RSD with acceptable limits of variance equal to 10%.

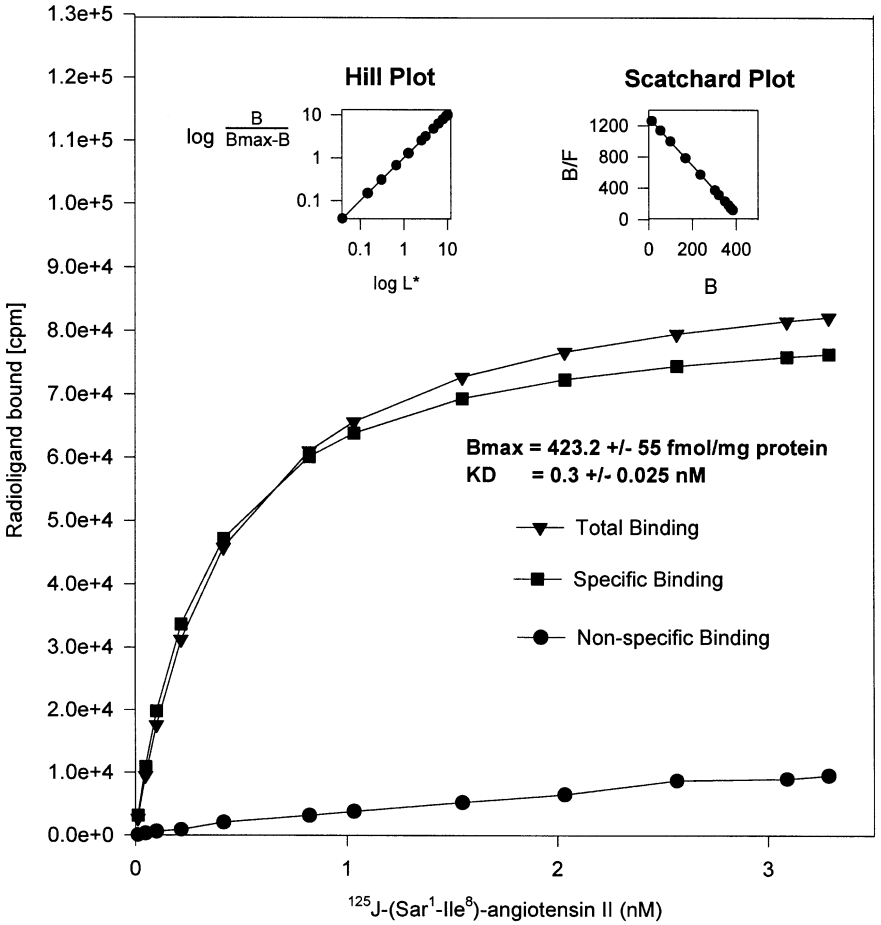

Fig. 2. Specific [^{125}J](Sar^{1}Ile^8)-angiotensin II binding (■) to rat lung homogenate as a function of increasing concentrations of the radioligand. Specific binding was determined experimentally as the difference between total and nonspecific binding in the absence and presence of 1 μM AII. Each point represents the average of three determinations. Inserts: Hill plot derived from the specific [^{125}J](Sar^{1}Ile^8)-angiotensin II binding data with the ordinate log $B/(B_{max}-B)$ representing [^{125}J](Sar^{1}Ile^8)-angiotensin II bound in fmol mg^{-1} protein, and the abscissa log L^* expressing free [^{125}J](Sar^{1}Ile^8)-angiotensin II unbound in nM. Scatchard Plot derived from the specific [^{125}J](Sar^{1}Ile^8)-angiotensin II binding data with the ordinate B/F representing bound over free [^{125}J](Sar^{1}Ile^8)-angiotensin II in fmol mg^{-1} protein nM^{-1}, and the abscissa B expressing [^{125}J](Sar^{1}Ile^8)-angiotensin II bound.

2.7.2. Limit of quantification

The limit of quantification was defined by the antagonist concentration which inhibited 10% of the radioligand binding to the angiotensin-AT$_1$ receptors with the radioligand being present in a final concentration of 0.06 nM. The precision criterion for the selection of the limit of quantification was that the RSD% does not exceed 15%.

2.7.3. Binding kinetics

The kinetics of ligand association and dissociation were investigated to establish the time frame required for equilibrium binding according to:

$$d[RL^*]/dt = k_{12}[R][L^*] - k_{21}[RL^*] \quad [18]$$

2.7.4. Incubation temperature

Incubations at 20, 25, 30 and 37°C yielded

Table 2
Intra- and interday variability

	losartan	EXP 3174	UP 269-6	SL 91.0102-90 DL
Intraday:				
Concentration range (ng ml^{-1})	0.01–50	0.01–50	0.01–50	0.01–50
Number of replicates	10	10	10	10
RSD (%)	5.0	4.4	7.2	6.8
Interday:				
Concentration range (ng ml^{-1})	0.01–50	0.01–50	0.01–50	0.01–50
Number of replicates	10	10	10	10
RSD (%)	9.2	8.6	8.8	7.5

comparable results suggesting that the influence of temperature was negligible in the working range.

2.7.5. Association kinetics

The speed of the receptor–ligand binding was analyzed in Hepes-buffer as well as in human plasma, as described in [18]. In brief, buffer (or plasma) and a constant radioligand concentration of 0.06 nM were placed into 96-well microtiter plates. The reaction was started by successively adding a constant amount of membrane suspension (50 μg protein per sample) in 5–10 min intervals up to 2 h. Consecutively, the samples were filtrated through presoaked Whatman GF/C glass fiber filters, and the tissue-bound radioactivity was measured by γ-counting. In this context, an excessive amount of free radioligand was applied to avoid ligand depletion. For the estimation of the association rate constant k_{12}, the specifically bound radioactivity in (fmol/mg protein), i.e. the totally bound minus the non-specifically bound, was semilogarithmically plotted versus time. Further data transformations [18] obtained the association rate constant k_{12}.

2.7.6. Dissociation kinetics

For the dissociation rate constant k_{21}, constant amounts of radioligand (0.06 nM) and receptor protein (50 μg protein per sample) were incubated for 2 h (including nsb samples). The receptor–ligand complex was then dissolved by gradual addition of losartan (1 μM) as an unlabelled competitor to the medium in 5–10 min intervals up to 2 h. The specifically bound radioligand was semilogarithmically plotted versus time, with the slope yielding the dissociation rate constant k_{21}.

2.7.7. Binding specificity

The binding specificity, i.e. the saturability of the receptor membranes, was verified by incubating different amounts of receptor protein (10, 25, 50, 75 and 100 μg protein per sample, in a total volume of 300 μl) with a constant radioligand concentration of 0.06 nM for 60 min at 25°C. After separation from unbound, bound radioligand was then plotted versus the amount of protein per sample.

3. Results and discussion

3.1. Validation

3.1.1. Intra- and interday variability

The average intra- and interday variability was below 10% in all cases (Table 2).

3.1.2. Limit of quantification

The limits of quantification which were defined as the respective antagonist concentration, which inhibited 10% of the radioligand binding, are listed in Table 3 for the determination in Hepes-buffer as well as in blank human or rat plasma. Based on the precision values, which were below 10% in all cases, the limits of quantification ranked between 1.6–9.1 pg ml^{-1} in Hepes-buffer and 0.11–1.17 ng ml^{-1} in blank plasma, respectively.

3.1.3. Binding kinetics

For the association of receptor–ligand binding, a rate constant k_{12} of 5.0×10^{7} M^{-1} s^{-1} was calculated implying that the reaction took place very rapidly. With a dissociation rate constant k_{21} of 2.0×10^{-2} M^{-1} s^{-1}, the ratio of k_{21}:k_{12} yielded an equilibrium dissociation constant K_D of approximately 0.4×10^{-9} M, which was slightly higher than the experimentally obtained value of $0.25–0.3 \times 10^{-9}$ M. For this value, an optimal incubation time of 60 min was derived according to [18].

3.1.4. Binding linearity

The correlation of bound radioligand concentration versus the amount of protein per sample revealed linear behaviour for all concentrations confirming binding specificity.

3.2. Identification of [^{125}J](Sar^{1}-Ile^{8})-angiotensin II binding sites in rat lung

Specific binding of [^{125}J](Sar^{1}-Ile^{8})-angiotensin II (0.025–3.5 nM) to rat lung appeared to be saturable, while the non-specific binding increased linearly with the ligand concentration (Fig. 2). In most experiments, non-specific binding did not exceed 15% of the total radioactivity bound. Non-linear curve-fitting revealed the existence of one high-affinity binding site for [^{125}J](Sar^{1}-Ile^{8})-angiotensin II with an apparent maximal binding capacity (B_{max}) of 423 ± 55 fmol mg^{-1} protein and an equilibrium dissociation constant (K_D) of 0.3 ± 0.025 nM (mean $\pm$ SD, $n = 3$). Scatchard as well as Hill analysis were performed to further test the assumption of a high-affinity binding site. The Hill coefficient (Fig. 2) of [^{125}J](Sar^{1}-Ile^{8})-angiotensin II was with 1.01 ± 0.03 very close to uniformity indicating a single class of binding sites. The Scatchard plot (Fig. 2) yielded a slope -1 ($= 1/K_D \rightarrow 1/(0.3 \pm 0.025)$) of approximately -3, confirming the existence of a single class of binding sites.

Table 3
Limit of quantification for losartan, EXP 3174, SL 91.0102-90 DL and UP 269-6 in Hepes-buffer (pg ml^{-1}) vs. blank plasma (ng ml^{-1})

Limit of quantification	Hepes-buffer (pg ml^{-1})	Blank plasma (ng ml^{-1})
losartan	8.7 ± 0.26	1.08 ± 0.030
EXP 3174	1.6 ± 0.06	0.11 ± 0.005
SL 91.0102-90 DL	9.1 ± 0.21	1.02 ± 0.024
UP 269-6	8.2 ± 0.19	1.17 ± 0.040

The values represent the mean $\pm$ SD of 3–5 determinations.

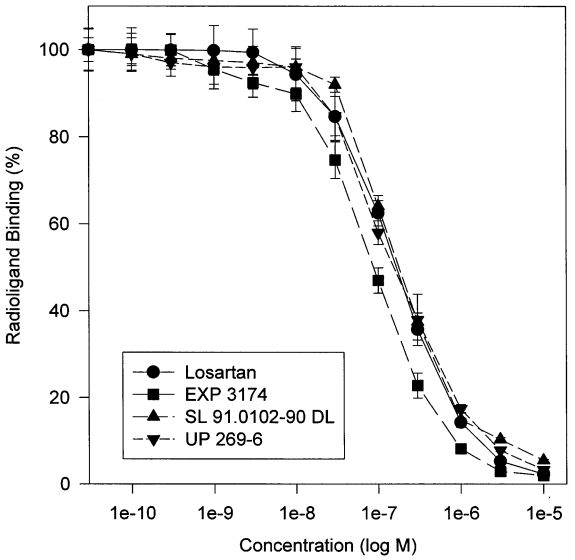

Fig. 3. Inhibition of specific [^{125}J](Sar^{1}Ile^{8})-angiotensin II binding to rat lung homogenate by losartan, EXP 3174, SL 91.0102-90 DL and UP 269-6 in human plasma. Displacement was determined by incubating the radioligand with $1e^{-10}$ to $1e^{-5}$ molar concentrations of each drug. The ordinate indicates the percentage of specific [^{125}J](Sar^{1}Ile^{8})-angiotensin II binding, the abscissa expresses the logarithmic molar concentration of the unlabelled antagonists. Each point represents the average of three determinations.

3.2.1. Characterization of the AT$_1$-affinity profiles of losartan, EXP 3174, UP 269-6 and SL 91.0102-90 DL

Unlabelled nonpeptide AT$_1$-antagonists (losartan, EXP 3174, SL 91.0102-90 DL and UP 269-6) were tested for their ability to compete with [^{125}J](Sar^{1}-Ile^{8})-angiotensin II for specific AT$_1$-receptor sites in rat lung homogenate. The displacement curves in blank plasma as well as the respective K_i values in protein-free Hepes-buffer compared to blank plasma are shown in Fig. 3 and Table 4. The correction of the K_i values

Table 4
Inhibition of specific [^{125}J](Sar^{1}Ile^8)-angiotensin II binding to rat lung homogenate by losartan, EXP 3174, SL 91.0102-90 DL and UP 269-6 in Hepes-buffer vs. blank plasma

K_i-values (nM)	Hepes-buffer	Blank plasma
Losartan	16 ± 1.1	278 ± 25
EXP 3174	1.1 ± 0.1	144 ± 14
SL 91.0102-90 DL	8 ± 0.5	125 ± 15
UP 269-6	10 ± 0.9	147 ± 15

Displacement studies were conducted as described in Figs. 3 and 4. The K_i values were calculated and described in Section 2. The values represent the mean $\pm$ SD of 3–5 determinations.

according to Cheng and Prusoff [19] yielded the corresponding IC_{50}-values (Table 5).

3.2.2. Plasma protein binding

The impact of plasma protein binding was tested for all antagonists investigated to evaluate their differences in the amounts of free, i.e. non protein-bound, drug concentration. Fig. 4 illustrates the striking difference in binding potency observed with EXP 3174 in protein-free Hepes-buffer compared to blank plasma. Although EXP 3174 was the most potent inhibitor of [^{125}J](Sar^1-Ile^8)-angiotensin II binding in Hepes-buffer, as demonstrated by K_i values of 1.1 ± 0.1 nM (Fig. 4), it did not prominently excel in human plasma compared to the parent compound losartan as well as SL 91.0102-90 DL and UP 269-6 (Fig. 3) due to extensive plasma protein binding (99.8%) [20]. Thus, plasma protein binding caused a remarkable decrease in receptor affinity by a factor of 120 for EXP 3174 ($\sim$1.1 versus $\sim$144 nM), contrasting with a factor of only 17 for the parent compound Iosartan ($\sim$16 versus $\sim$278 nM).

Table 5
IC_{50} values of losartan, EXP 3174, SL 91.0102-90 DL and UP 269-6 following correction of their K_i-values (Table 4) according to Cheng and Prusoff [19]

Ic_{50} values (ng ml^{-1})	Hepes-buffer	Blank plasma
Losartan	19.2 ± 1.32	333.6 ± 27.0
EXP 3174	1.3 ± 0.12	172.8 ± 15.8
SL 91.0102-90 DL	9.6 ± 0.60	150.0 ± 8.2
UP 269-6	12.0 ± 1.08	176.4 ± 13.2

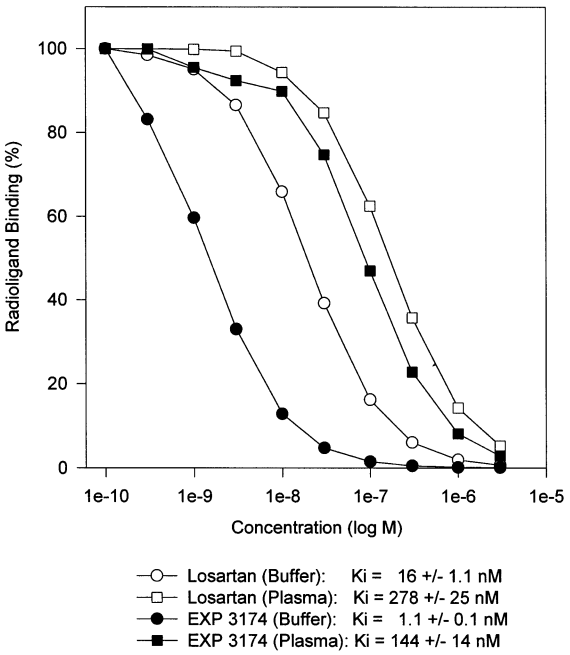

Fig. 4. Inhibition of specific [^{125}J](Sar^{1}Ile^8)-angiotensin II binding to rat lung homogenate by EXP 3174 and losartan in Hepes-buffer vs. human plasma (with ordinate and abscissa described in Fig. 2). Each point represents the average of three determinations.

In plasma, losartan proved to be half as potent as its metabolite with an average K_i value of 278 nM and an IC_{50} value of 333.6 ng ml^{-1} compared to approximately 144 nM and 172.8 ng ml^{-1} for EXP 3174. In comparison, UP 269-6 and SL 91.0102-90 DL yielded average K_i values of 147 and 125 nM and IC_{50}-values of 176.4 and 150 ng ml^{-1}, respectively, thus ranging between losartan and EXP 3174.

3.2.3. Ex-vivo/in-vitro analysis of AT_1-receptor occupancy by angiotensin-AT_1-antagonists versus radioligand in biological samples

The efficacy of the antagonists investigated to reduce radioligand binding was further studied by ex-vivo/in-vitro analysis of biological samples.

3.2.3.1. Following intraperitoneal administration of losartan to rats (Section 2.4a). Fig. 5 shows the time-course of radioligand binding in rat plasma up to 36 h post dose. Maximum inhibition was

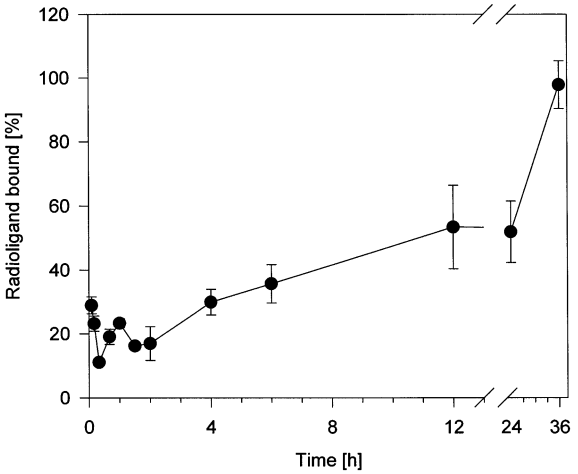

Fig. 5. Radioligand binding (%) 5, 10, 20, 40 min and 1, 1.5, 2, 4, 6, 12, 24 and 36 h following intraperitoneal administration of losartan (3 mg kg^{-1} body weight) to rats (mean values ± SD for $n = 4$ for each time point).

achieved 20 min after administration by reducing the radioligand binding to approximately 10% of its value in the absence of any drug. This strong angiotensin-II antagonistic effect, which was mostly due to the rapid formation of EXP 3174, was observed up to 6 h post dose, where radioligand binding was still substantially decreased to about 35%. At 12 h post dose, radioligand binding was half maximal and did not further increase until 24 h post dose. Thereafter, the antagonistic effect gradually vanished and was completely abolished at 36 h post dose.

3.2.3.2. Following oral administration of UP 269-6 to healthy volunteers. Fig. 6 illustrates the time-course of radioligand binding (%) in human plasma before drug administration and 1, 4, 10 and 24 h post dosing according to Section 2.4b. With all doses, maximum inhibition of radioligand binding was achieved within the first hour post dose, rapidly levelling out following the 5 or 10 mg doses and more prolonged—up to 10 h post dose—following the higher doses of 20–180 mg.

3.2.3.3. Following oral administration of SL 91.0102-90 DL to healthy volunteers. Fig. 7 demonstrates the time-course of radioligand binding (%) in plasma prior and 2, 4, 6, 10 and 24 h after drug administration. With all doses, the maximum inhibition of radioligand binding was achieved at (or before) 2 h post dose, gradually levelling out in a clearly dose-dependent manner.

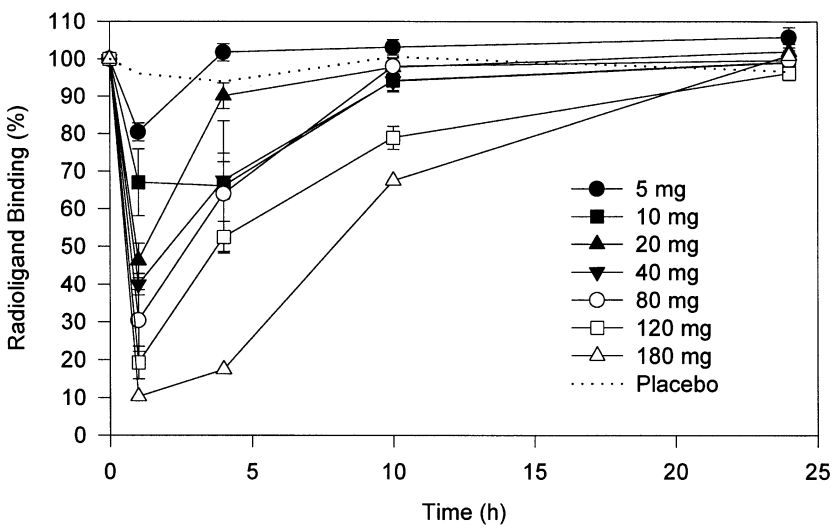

Fig. 6. Radioligand binding (%) 0, 1, 4, 10 and 24 h following the oral administration of 5–180 mg of UP 269-6 (mean values ± SD).

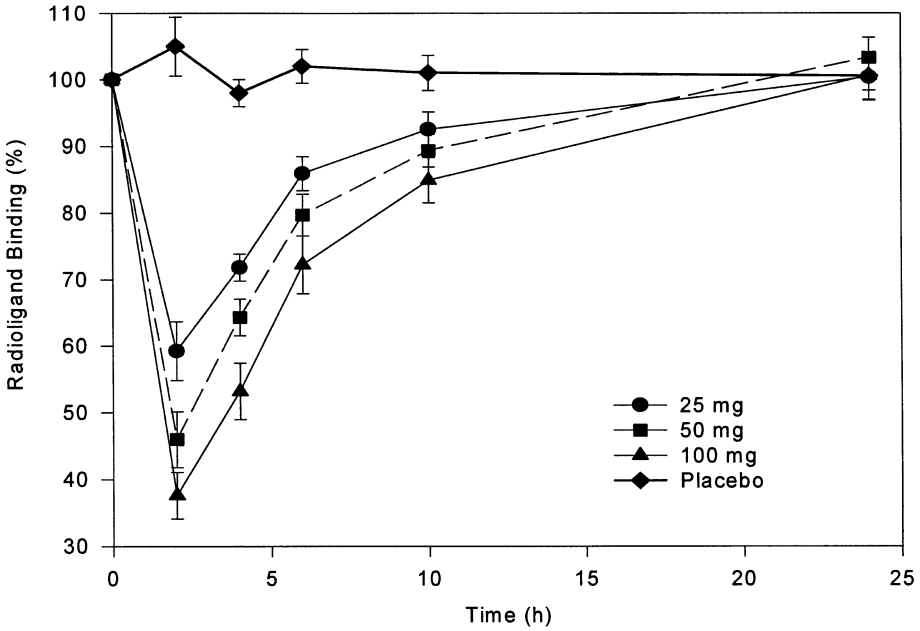

Fig. 7. Radioligand binding (%) 0, 2, 4, 6, 10 and 24 h following the oral administration of 25, 50 or 100 mg of SL 91.0102-90 DL (mean values ± SD).

3.2.4. HPLC analysis of biological samples

The respective plasma drug levels for all antagonists analyzed reflected the time-course of receptor occupancy described above (data not shown). While losartan as well as EXP 3174 plasma concentrations were found up to 24 h post dose, both UP 269-6 and SL 91.0102-90 DL plasma levels were only detectable up to 10 h post dose.

3.2.5. Correlation of radioreceptor assay and HPLC data (obtained from the ex-vivo/in-vitro radioligand binding studies and HPLC analysis described above)

While HPLC data alone neither allow the assessment of pharmacodynamic effects nor the estimation of the therapeutically relevant drug concentration range, RRA data represent a criterion to define receptor blockade in vitro and, thus, to predict receptor blockade in vivo.

3.2.5.1. Losartan and EXP 3174 concentration equivalents. The correlation of the RRA data with the HPLC concentrations of losartan alone revealed a considerably higher amount of concentration equivalents from RRA than from HPLC. Moreover, the time sequence of points followed a counterclockwise hysteresis loop, i.e. more effect was observed with the same losartan HPLC concentrations at later points, strongly indicating the formation of one or more active metabolites with an increase of metabolite:parent compound ratio with time (Fig. 8, left side) [21]. In contrast, the correlation of the RRA concentration equivalents and the total HPLC concentrations of both losartan and EXP 3174 yielded close agreement with data points scattering around the identity line (Fig. 8, right side). These results confirmed the findings of Stearns et al. [22] that the angiotensin-II antagonistic effect following losartan administration was mediated to a major part by EXP 3174, obviously without contribution of any other metabolites.

3.2.5.2. UP 269-6 concentration equivalents. The correlation of the RRA and HPLC data from the studies described yielded data points that scattered around the identity line indicating close agreement of both data sets. Fig. 9 shows a

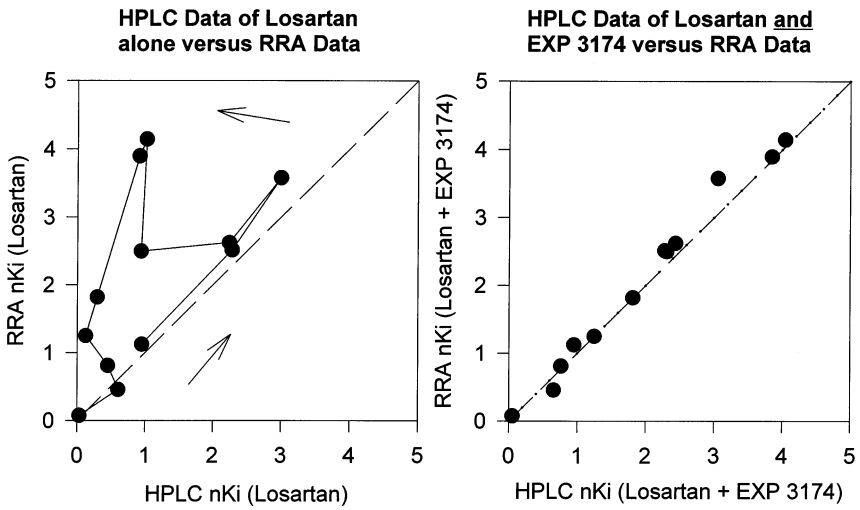

Fig. 8. Correlation of RRA and HPLC data: Left side, correlation with HPLC data from losartan alone; Right side, correlation with HPLC data from losartan and EXP 3174.

comprehensive picture of the correlation of RRA and HPLC data over the entire dosing range of 5–180 mg.

3.2.5.3. SL 91.0102-90 DL concentration equivalents. In contrast to UP 269-6, which seems to be exclusively responsible for the angiotensin-II antagonistic effect as described above, active metabolites of SL 91.0102-90 DL most probably contribute to the observed effect, since with all doses a considerably higher amount of concentration equivalents from RRA was found when correlated with the respective HPLC data (Fig. 10).

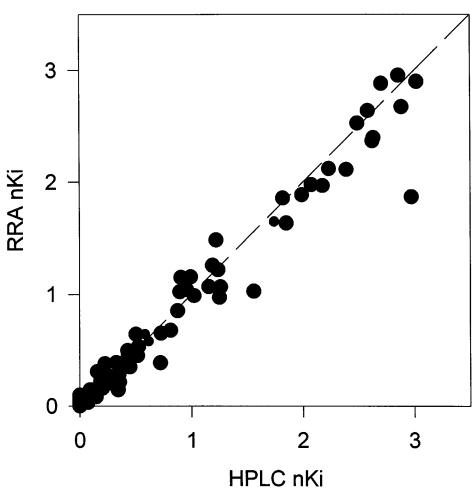

Fig. 9. Correlation of RRA and HPLC data (for 5–180 mg of UP 269-6).

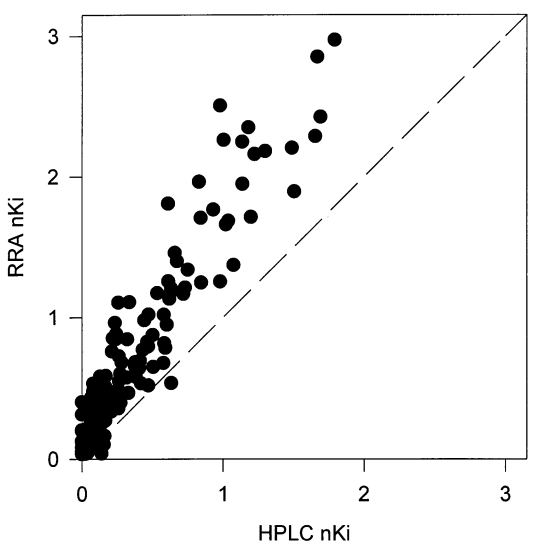

Fig. 10. Correlation of RRA and HPLC data (for 25, 50 or 100 mg of SL 91.0102-90 DL).

4. Conclusion

A reliable and sensitive radioreceptor assay was developed and validated to determine the angiotensin-II-AT_1 antagonistic profiles of losartan and its main active metabolite EXP 3174 by in vitro and ex-vivo/in-vitro radioligand binding studies. In addition, the method proved to be appropriate for the analysis of congeners of losartan, exemplified by UP 269-6 and SL 91.0102-90 DL. The comparative performance of displacement studies in blank plasma versus protein-free Hepes-buffer allowed the determination of each drug's tendency to bind to plasma proteins.

The correlation of normalized RRA (for each drug separately) as well as HPLC data from losartan, UP 269-6 and SL 91.0102-90 DL as the respective parent compounds revealed highly interesting results. While the correlation of UP 269-6 data yielded measure points that scattered around the identity line indicating congruence of data sets, the correlation of the respective SL 91.0102-90 DL data suggested the presence of significant amounts of active metabolites, since considerably higher amounts of concentration equivalents were found resulting from RRA than from HPLC.

The correlation of losartan RRA and HPLC data exhibited a picture similar to SL 91.0102-90 DL indicating that the antagonistic potency of losartan was based on the contribution of one or more active metabolites. The subsequent correlation of RRA concentration equivalents with the total HPLC concentration equivalents of both losartan and EXP 3174 yielded very close agreement confirming that only losartan and EXP 3174 exert the angiotensin-II antagonism without further contribution of metabolites.

In conclusion, the reported comparative analysis by both RRA and HPLC allows evaluation of the potential contribution of active metabolites to the observed angiotensin-II antagonistic effect.

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