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In vitro pharmacological characterization of a new selective angiotensin AT_1 receptor antagonist, UR-7280

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Abstract

UR-7280 (3-*tert*-butyl-1-propyl-5-[[2′-(1*H*-tetrazol-5-yl)-1,1′-biphenyl-4-yl]methyl]-1*H*-pyrazole-4-carboxylic acid) is a new and potent angiotensin AT_1-selective receptor antagonist. Binding studies in rat liver membranes showed that UR-7280 is an apparently competitive antagonist. However, in rabbit aorta this compound antagonized the angiotensin II-induced contractile response in an insurmountable way, causing a significant reduction of the maximal response. Additional binding studies evidenced that UR-7280 has a slowly reversible binding profile, consistent with its functional properties in rabbit aorta. The results obtained with a series of structural analogues of UR-7280 demonstrated a relationship between the size of the pyrazole 3-substituent and the surmountable or insurmountable mode of antagonism, indicating that this position may play a key role in the interaction between the antagonist and the angiotensin AT_1 receptor.

Keywords: Angiotensin AT_1 receptor; Angiotensin II; Binding; Losartan; Aorta, rabbit; UR-7280

1. Introduction

The angiotensin II receptor is the final site of action in the renin-angiotensin system and has become the most common target in the development of new treatments for hypertension (Wexler et al., 1996; Goodfriend et al., 1996). Two subtypes of angiotensin II receptors have been reported (Birabeau et al., 1984). Subtype 1 (AT_1) is responsible for the main biological effects of angiotensin II, such as vasoconstriction, aldosterone release, cellular proliferation or water intake. The role of the subtype 2 receptor (AT_2) is unknown (Nahmias and Strosberg, 1995), although it appears to counteract the haemodynamic effect of the AT_1 receptor and may be involved in the regulation of some central nervous system functions (Ichiki et al., 1995). Since losartan, the first non-peptide orally active angiotensin II receptor antagonist, was described (Chiu et al., 1990), a number of non-peptide selective angiotensin AT_1 receptor antagonists have been synthesized and characterized as having an orally active antihypertensive effect in several animal models (Smith et al., 1992). Some of these newly synthesized compounds have been described in functional studies as insurmountable angiotensin II receptor antagonists (Panek et al., 1995). They shift the dose-response curves with a simultaneous decrease in the maximum achievable response. In binding studies, however, some of these compounds display a competitive behaviour, decreasing the affinity of the agonist for the receptor with no effect on the number of binding sites. The explanation for this apparent contradiction remains elusive (Reitz et al., 1993; Olins et al., 1995).

UR-7280 (3-*tert*-butyl-1-propyl-5-[[2′-(1*H*-tetrazol-5-yl)-1,1′-biphenyl-4-yl]methyl]-1*H*-pyrazole-4-carboxylic acid, Fig. 1) is a potent and highly selective angiotensin AT_1 receptor antagonist synthesized in our laboratory (Almansa et al., submitted). The aim of the present study was to determine the pharmacological profile that might account for the observed insurmountable antagonism by UR-7280, in comparison to losartan, a well-characterized surmountable angiotensin II receptor antagonist. To this end, we also tested a set of structural analogues of UR-7280 with different substituents at the 3-position of the pyrazole

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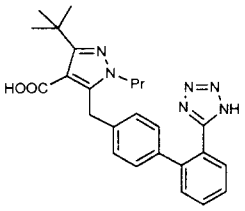


Fig. 1. Chemical structure of UR-7280.

ring to establish the significance of this position for the mode and potency of antagonism by these compounds.

2. Materials and methods

2.1. Materials

Angiotensin II, [Sar^1,Ala^8]angiotensin II (saralasin) and phenylmethylsulfonyl fluoride were purchased from Sigma (st. Louis, MO, USA) [^{3}H]Angiotensin II (71 Ci/mmol) was obtained from New England Research Products (Dreieich, Germany). Losartan and valsartan were kindly supplied by DuPont-Merck Pharmaceutical Company (Wilmington, DE, USA) and Ciba-Geigy (Basel, Switzerland), respectively. EXP3174 ((2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl) methyl] imidazole-5-carboxylic acid), MK-996 (*N*-((4′-((5,7-dimethyl-2-ethyl-3*H*-imidazol[4,5-*b*]pyridin-3-yl)methyl)(1,1′-biphenyl)-2-yl)sulfonylbenzamide) and UR-7280 and its structural analogues were synthesized in the Chemistry Department of J. Uriach and co-workers.

2.2. Preparations of membranes

Angiotensin II receptors from rat liver and adrenal microsomes were prepared by modifications of previously described methods (Chiu et al., 1990). Tissues were obtained after cervical dislocation, and were collected in 50 mM Tris-HCl buffer, pH 7.5, containing 0.05 mg/ml of bacitracin and 0.1 mM phenylmethylsulfonyl fluoride, so that the concentration was 2% (w/v). The mixture was homogenized and the homogenate was centrifuged at 1000 $\times g$ for 10 min; the supernatant was further centrifuged at 100 000 $\times g$ for 1 h. The resultant membrane pellet was then resuspended (1 g of wet weight/ml) in the above buffer and a protein concentration of 23.5 $\pm$ 0.8 and 16.1 $\pm$ 0.2 mg protein/ml for liver and adrenal membranes, respectively, was obtained. Aliquots (700 μl) of this suspension were stored frozen at −70°C until used.

2.3. Radioligand binding assays

Membranes were diluted to a final volume of 20 ml in incubation buffer containing (final concentrations): NaCl (120 mM), $MgCl_2$ (5 mM), 0.006% bovine serum albumin, and Tris (50 mM), adjusted to pH 7.5. To determine AT_2 binding, rat adrenal membranes were diluted in the same way, but with 5 mM of dithiothreitol included in the incubation buffer. Incubation was started by adding 2 nM [^{3}H]angiotensin II. Total incubation volume was 250 μl with a final protein concentration of approximately 0.7 mg protein/ml (liver membranes) or 0.4 mg protein/ml (adrenal membranes). Non-specific bindig was measured by incubation in the presence of 10 μM of non-labelled angiotensin II. Test compounds were studied in the 10^{-5} to 10^{-12} M range of concentrations. Binding was stopped by rapid filtration through GF/C glass fiber filters using a Millipore Multiscreen device. Filters were washed three times with 0.25 ml of the incubation buffer. Dry filters were placed in vials containing 3 ml of scintillation fluid and the radioactivity was counted in a scintillation counter.

Scatchard analysis (Scatchard, 1949) was applied using the aforementioned protocol in the presence and absence of UR-7280 (1 and 10 nM). In this case, a concentration range of 0.1–15 nM of [^{3}H]angiotensin II was used.

2.4. Functional studies in rabbit aorta

Helical strips of thoracic aorta from male New Zealand White rabbits were kept in an organ bath at 37°C in a solution containing 154 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 6 mM $NaHCO_3$ and 11 mM glucose, constantly aerated (95% O_2/5% CO_2). The isometric contraction with a loading tension of 2 g was recorded with a strain gauge transducer (Letica, TRI 112) connected to a recorder (Letica, Polygraph 4006). The strips were left to stabilize for 90 min and then a cumulative dose-response curve with angiotensin II (10^{-11} to 10^{-4} M), noradrenaline (10^{-10} to 10^{-4} M), 5-hydroxytryptamine (5-HT, 10^{-9} to 10^{-4} M) or potassium chloride (10–70 mM) was made, adding vasoconstrictor agents to the bath when the maximum contraction induced by a prior concentration was reached. The highest response to angiotensin II was considered the maximal response. Then, the strips were washed by superfusion for an hour, at intervals of approximately 1 min, until return to the baseline. Test compounds were added 30 min (or 3 h, in the indicated cases) before the next concentration-response curve was done.

2.5. Association and dissociation studies

The association and dissociation rates were roughly estimated as described previously (Hara et al., 1995). To determine the association rate, the membranes were preincubated with the antagonist for the indicated times and then further incubated for 20 or 60 min after the addition of 2 nM of [^{3}H]angiotensin II. To estimate the dissociation rate, the membranes were pre-incubated with the antagonist for 45 min, washed twice with incubation buffer by

centrifugation at 100 000 × g for 30 min at 4°C, and then incubated for a period of 60 or 80 min after the addition of 2 nM of [^{3}H]angiotensin II.

To determine how the preincubation time with UR-7280 affects the antagonist-receptor interaction, rat liver membranes were preincubated (45 min) with 5 nM UR-7280 or vehicle and then washed thoroughly (100 000 × g, 30 min, 4°C). Then, the membranes were incubated for 30 or 120 min with various concentrations of [^{3}H]angiotensin II (0.1–15 nM) and the data were analyzed according to Scatchard (1949).

In each case, 10 μM of non-labelled angiotensin II was used to determine non-specific binding.

2.6. Data analysis

Results from the binding studies are expressed as the means ± S.E.M. of 2–5 independent experiments using different membrane preparations. The data were evaluated by means of the non-linear curve fitting program LIGAND (Munson and Rodbard, 1980).

Responses in functional studies with rabbit aorta were expressed as percentages of the maximal previous angiotensin II concentration. The apparent pA_2 values were obtained from the Schild equation for surmountable antagonists (Tallarida and Murray, 1981). For insurmountable antagonists an apparent K_B was calculated as [antagonist]/[slope − 1], where slope was obtained by dual reciprocal regression analysis of 1/[A] versus 1/[A'] in the absence and presence of antagonist, respectively (Kenakin, 1993).

3. Results

3.1. Binding studies

UR-7280 displayed high specific affinity for rat liver and adrenal angiotensin AT_1 receptor (K_i = 1.6 ± 0.2 nM and 1.9 ± 0.8 nM, respectively; Table 1) with no effect on binding to the rat adrenal and cerebellar AT_2 subtype (data not shown). The potency of UR-7280 at the AT_1 receptor in rat liver was approximately 10-fold higher than that of losartan and was of the same order as that of EXP3174, valsartan, and MK-996 (Table 1).

Table 1
Binding affinity (K_i) of several antagonists for rat adrenal and liver angiotensin AT_1 receptor

Compound	K_i (nM)	
	Adrenals	Liver
Losartan	15.3 ± 8.0	16.9 ± 2.8
Valsartan	6.8 ± 4.8	2.7 ± 0.3
MK-996	1.8 ± 0.4	2.1 ± 0.5
EXP3174	2.1 ± 0.3	1.4 ± 0.05
UR-7280	1.9 ± 0.8	1.6 ± 0.2

Values are the means ± S.E. of 2–5 separate experiments performed in duplicate and were calculated by means of the non-linear fit program LIGAND (Munson and Rodbard, 1980).

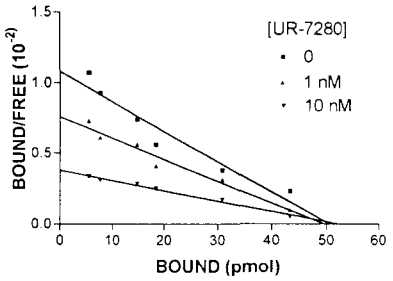


Fig. 2. Scatchard analysis of [^{3}H]angiotensin II saturation binding in rat liver membranes in the presence of UR-7280. Data points are the means of duplicate determinations and are representative of three separate experiments. The lines were drawn by regression analysis.

Scatchard analysis of saturation binding with increasing concentrations of UR-7280 revealed competitive inhibition of [^{3}H]angiotensin II to AT_1 receptors in rat liver membranes (Fig. 2).

3.2. Effect on vascular smooth muscle contraction of rabbit aorta

In tests of UR-7280 ability to antagonize angiotensin II-induced contraction in spiral strips of isolated rabbit aorta, the angiotensin II-induced contraction curve was shifted in parallel to the right with a significant decrease (of approximately 30% of the control value) in maximum contractile response (Fig. 3). UR-7280 behaves as an

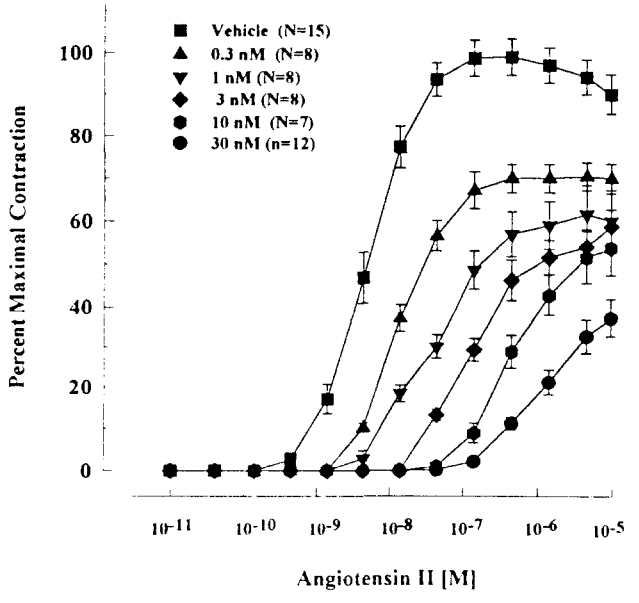


Fig. 3. Effect of cumulative angiotensin II-induced contraction on isolated rabbit aortic strips in the absence and presence of UR-7280 (0.3–30 nM) incubated for 30 min. Results are shown as mean responses ± S.E.M.

insurmountable antagonist as defined by Gaddum et al. (1955), and only an apparent pK_b value could be calculated ($pK_b = 10.2 \pm 0.36$, slope $= 1.31 \pm 0.31$). Similar assays were carried out after 30 min or 3 h of incubation with 1 nM or 10 nM of antagonist with no differences observed between doses or times (data not shown), suggesting that UR-7280 reaches equilibrium with the receptor before 30 min.

To further characterize the nature of the insurmountable antagonism of UR-7280, we performed receptor protection experiments by incubating this compound simultaneously with the surmountable antagonist losartan (Wong and Timmermans, 1991). With 30 nM UR-7280 the maximal contractile force in response to angiotensin II was restored from 40% of the control value in the absence of losartan, to 75% of the control values when the experiments were carried out in the presence of 0.1, 1 or 10 μM losartan (Fig. 4), suggesting that UR-7280 and losartan interact with the same binding site.

In assays performed in the presence of 0.1% bovine serum albumin to test for a possible interaction between UR-7280 and serum proteins, the apparent pK_b of UR-7280 fell from the initial value of 10.2 to 7.0, suggesting high binding of UR-7280 to bovine serum albumin. In both cases, insurmountable behaviour was maintained. Similar results had been reported for structurally related compounds (Robertson et al., 1992; Dickinson et al., 1994). The pronounced binding of these molecules to plasma and tissue proteins may account, almost in part, for the long duration of action of all these angiotensin II receptor antagonists in vivo.

UR-7280 did not show any effect on the contractile response to KCl, noradrenaline and serotonin even at a

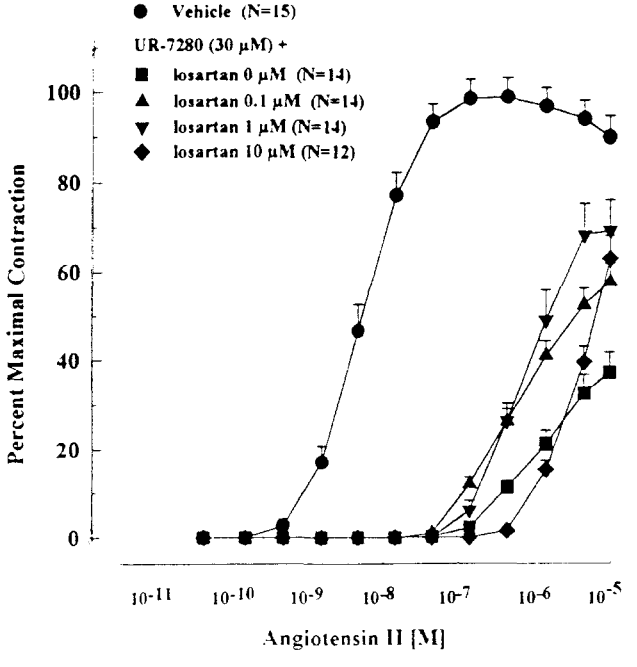


Fig. 4. Effect of UR-7280 (30 nM) on cumulative angiotensin II-induced contraction on isolated rabbit aortic strips incubated for 30 min in the absence and presence of losartan (0.1, 1 and 10 μM). Results are shown as mean responses ± S.E.M.

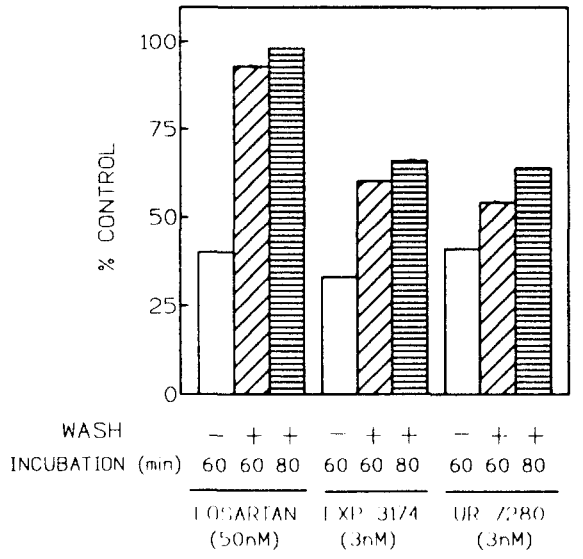


Fig. 5. Effects of losartan, EXP3174 and UR-7280 on the [^{3}H]angiotensin II binding to rat liver membranes before and after washout of the antagonists. Open columns: membranes were preincubated with each antagonist for 45 min and then further incubated for 60 min with [^{3}H]angiotensin II (2 nM). Hatched columns: after preincubation with the antagonists, the membranes were washed twice for centrifugation (100000× g, 30 min) and incubated with 2 nM of [^{3}H]angiotensin II for 60 (cross hatched) or 80 min (horizontal hatched). The results are expressed as percentage of binding with respect to controls preincubated without the antagonist. Data points are mean values from two separate experiments.

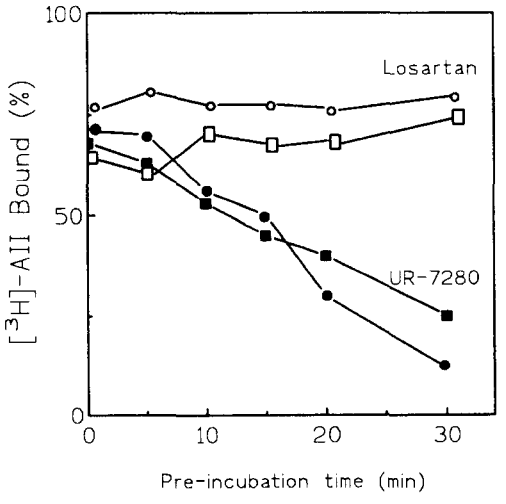


Fig. 6. Time course for the effect of preincubation with UR-7280 and losartan on [^{3}H]angiotensin II binding to rat liver membranes. Membranes were preincubated with 10 nM losartan or 1 nM UR-7280 for the periods indicated. Incubations were continued for another 20 min (○, ●) or 60 min (□, ■) after the addition of [^{3}H]angiotensin II (2 nM). Data points are the means of two separate experiments.

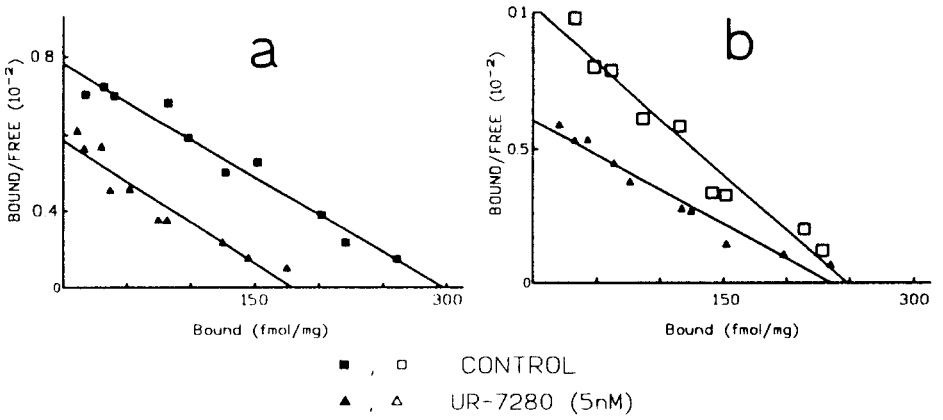


Fig. 7. Effect of UR-7280 on Scatchard analysis of [^{3}H]angiotensin II binding to rat liver membranes. Membranes were preincubated (45 min) with or without 5 nM UR-7280 and were washed twice for centrifugation (100 000 × g, 30 min). The washed membranes were incubated with [^{3}H]angiotensin II for 30 min (a) or 120 min (b), and the amount of specific binding was determined. Data points are the means of two determinations; the experiment was repeated twice and similar results were obtained.

high concentration of 10^{-5} M, suggesting specific angiotensin II antagonism for this compound.

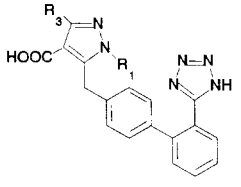
3.3. Association and dissociation of UR-7280

Fig. 5 shows a comparison (as percentage of controls incubated without the antagonists) of the dissociation rates of UR-7280, losartan and EXP3174 in rat liver membranes. [^{3}H]Angiotensin II binding to membranes pretreated with losartan reached the same level as it did with membranes preincubated without antagonist, whereas in the membranes pretreated with UR-7280 or EXP3174 this binding level was never achieved, even with incubation times up to 80 min.

Fig. 6 shows how the preincubation time with losartan or UR-7280 affected their potency. In the case of UR-7280, the longer the preincubation time, the stronger was the inhibition of [^{3}H]angiotensin II binding and this effect was more pronounced when an incubation time of 20 min was used. Preincubation, however, did not increase the potency of losartan at any of the times tested.

When angiotensin II and UR-7280 were added simultaneously (without preincubation of the membranes with the antagonist), UR-7280 behaved as a competitive antagonist as shown by the Scatchard analysis (Fig. 2). However, when the membranes were preincubated (45 min) with 5 nM UR-7280 before the addition of [^{3}H]angiotensin II, the B_{max} fell to 58 ± 4% of the control (Fig. 7a). If binding of the antagonist had been irreversible, this B_{max} reduction should have been observed even with longer incubation of

Table 2
K_i, pK_b values and mode of antagonism of a series of structural analogues of UR-7280



Analogue	R_3	R_1	K_i (nM)	pK_b	Mode [a]
1a	CH_3	n-C_3H_7	2.4	9.9	S
1b	C_2H_5	n-C_3H_7	0.9	11.2	I
1c	$CH(CH_3)_2$	n-C_3H_7	1.5	11.4	I
1d	CyPr [b]	n-C_3H_7	1.4	11.0	I
1e	n-C_4H_9	n-C_3H_7	1.6	10.2	I
1f	CH_2Ph	n-C_3H_7	1.2	9.4	I
2a	H	n-C_4H_9	2.5	9.5	S
2b	CH_3	n-C_4H_9	0.6	9.4	S
2c	C_2H_5	n-C_4H_9	1.5	11.3	I
2d	$CH(CH_3)_2$	n-C_4H_9	1.6	10.9	I
2e	n-C_3H_7	n-C_4H_9	2.6	9.7	I

[a] S = surmountable; I = insurmountable. [b] Cyclopropyl.

the membranes with [^{3}H]angiotensin II; however, when the incubation period was increased to 120 min, the B_{max} recovered to 90 ± 3% of the control value (Fig. 7b).

3.4. Relationships between 3-position pyrazole ring substituents and antagonistic behaviour

Table 2 shows the affinity (expressed as K_i and pK_b values) and the surmountable or insurmountable behaviour (expressed as the percentage of the maximum contractile response in control assays) of a set of 11 structural analogues of UR-7280 synthesized in our laboratory (Almansa et al., submitted). No relationship between affinity and surmountability could be observed, but there seems to be a relation between the length of the substituent at the 3-position of the pyrazole ring and the mode of antagonism of the compounds, such that for substituents larger than methyl the antagonism becomes insurmountable.

4. Discussion

UR-7280 behaves as a competitive angiotensin AT_1-specific antagonist, much like other AT_1 nonpeptide ligands, such as CV11974 (2-ethoxy-1-[[2′-1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid), SRL1080227 (6-propyl-7-oxo-4[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid) (Hara et al., 1995), valsartan (Criscione et al., 1993) and MK-996 (Chang et al., 1994). In functional studies in vascular smooth muscle UR-7280 displaced the concentration-response curves in a parallel fashion, indicating a competitive mode and, however, it also produced a 30% decrease in the maximal contraction response measured (insurmountable antagonism). These results are quite similar to those obtained with other angiotensin AT_1 receptor antagonists such as valsartan (36% decrease; Criscione et al., 1993), BMS-180560 (2-butyl-4-chloro-1-[[1-[2-(2*H*-tetrazol-5-yl)phenyl]-1*H*-indol-4-yl]methyl]-1*H*-imidazole-5-carboylic acid) (30% decrease; Dickinson et al., 1994) and SB 203220 ([*E*-α-[[2-butyl-1-(4-carboxy-1-naphthalenyl)methyl]-1*H*-imidazol-5-yl]-methylene]-2-thiophene-propanic acid]) (35% decrease; Aiyar et al., 1995).

An interesting finding of our study was the apparent relationship between the size of the substituent at the 3-position of the pyrazole ring and insurmountability (Table 2). Thus, when the substituent was hydrogen or methyl, the compounds behaved as surmountable antagonists, whereas in the case of larger substituents the antagonist was insurmountable. In fact, a relationship between the alkyl substituents of a phenyl ring at the 3-position of a related series of imidazol-2-ones and the type of antagonism has also been reported (Reitz et al., 1993), indicating that this position may play a key role in the interaction with the receptor.

The insurmountable behaviour of these compounds is thought to be due to the slow dissociation of the antagonist-receptor complex (Robertson et al., 1992; Hara et al., 1995). In keeping with this theory, the results obtained in our radioligand assays with UR-7280 show that this antagonist has a slow rate of association with the receptor binding site (Fig. 6) and hardly dissociates from it once it binds (Fig. 5). These slow association and dissociation rates are consistent with the functional properties of UR-7280 in rabbit aorta (where the antagonist is in contact with the receptor for 30 min before the angiotensin II is added). Moreover, treatment of liver membranes with UR-7280 for 45 min reduced the number of [^{3}H]angiotensin II binding sites (Fig. 7a), a result that is consistent with results of the aorta studies. This effect is not due to irreversible binding because, when the incubation time was prolonged (120 min, Fig. 7b), the receptor density increased again. All the aforementioned results agree with those reported for other insurmountable antagonists, such as SC-54629 ([1-(2,6-dimethylphenyl)-4-butyl-1,3-dihydro-3-[[6-[2-(1*H*-tetrazol-5-yl)phenyl]-3-pyridinyl]methyl]-2*H*-imidazol-2-one]) (Olins et al., 1995) and CV11974 (Hara et al., 1995) and according to these authors are most likely due to slow reversibility or pseudoirreversibility of binding.

Several molecular mechanisms have been put forth to explain this slow dissociation rate (see Wienen et al., 1992 or Panek et al., 1995). One is that insurmountable antagonists may bind to two different sites (Wong and Timmermans, 1991; Dickinson et al., 1994), a hypothesis further supported by receptor mutagenesis studies, which have helped to identify overlapping but distinct sites located in AT_1 transmembranal segments for competitive and insurmountable antagonists (Schambye et al., 1994). Other alternative explanations are the presence of an angiotensin AT_1 receptor subpopulation in rabbit aorta (Wienen et al., 1992) or a lipophilic pocket in the angiotensin II receptor which stabilizes the binding of the insurmountable inhibitors (Reitz et al., 1993; Noda et al., 1995).

In summary, the insurmountable mode of antagonism of the potent, highly selective angiotensin AT_1 receptor antagonist, UR-7280, seems to be related to the length of the substituent at the 3-position of the pirazole ring, perhaps because this affects the possibility of establishing additional interactions with other binding sites in the same receptor, or with a lipophilic pocket in the protein structure. These additional interactions may explain the slow reversibility of binding observed in radioligand studies, which in turn accounts for UR-7280's effective, long-lasting oral activity observed in vivo (Gómez et al., 1996).

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