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Properties of [^{3}H]LF 7-0156, a New Nonpeptide Antagonist Radioligand for the Type 1 Angiotensin II Receptor

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SUMMARY

LF 7-0156 (2-[[[2-butyl-1-[(4-carboxyphenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoic acid) is a nonpeptide angiotensin II receptor antagonist selective for the type 1 angiotensin receptor. In rabbit aortic rings, LF 7-0156 competitively antagonized angiotensin II-induced contractile responses, with a pA_2 value of 8.44. The synthesis of the radiolabeled compound [^{3}H]LF 7-0156 has allowed direct binding studies with several membrane or cell preparations. Consistent with competition experiments, the binding of [^{3}H]LF 7-0156 to purified rat liver membranes was characterized by a K_d value of 12.6 nM and very low pseudospecific or nonspecific binding; this latter characteristic confers to this compound an advantage over the structurally different compound [^{3}H]DuP 753, which is the only commercially available nonpeptide radioligand. [^{3}H]LF 7-0156 also bound to the type 1A angiotensin receptor expressed in Chinese hamster ovary cells, with high affinity (K_d = 3.5 nM) and a total absence of nonspecific binding. Functional antagonism in this cell system was assessed by the ability of LF 7-0156 to reverse angiotensin II-induced inositol phosphate production. These properties make [^{3}H]LF 7-0156 an interesting pharmacological tool and should allow future evaluation of recognition of the nonpeptide ligand by mutated receptors expressed in Chinese hamster ovary cells; it will facilitate the analysis of possible differences in receptor amino acids involved in the binding of peptide and nonpeptide ligands, as well as the extent of spatial overlap between several nonpeptide antagonists displaying different structural properties.

Recently, two AII receptor subtypes have been identified based on their relative affinities for the selective nonpeptide antagonists DuP 753 (Exp 89) and Exp 655 (PD123177) or its structural analogue WL-19 and the pseudopeptide antagonist CGP 42112A (1-3). The receptor subtype that displays high affinity for DuP 753 and weak affinity for Exp 655 or WL-19 and CGP 42112A has been designated as AT_1 (4) and is associated with most of the known functional responses of AII, including its major role in the regulation of blood pressure. The cloning of a cDNA encoding this AT_1 receptor has been reported (5, 6). The other receptor subtype, termed AT_2, is sensitive to Exp 655 or WL-19 and CGP 42112A, while showing no affinity for DuP 753. There exist a series of nonpeptide compounds exhibiting affinity for both AT_1 and AT_2 subtypes (7, 8). Although the primary sequence of the AT_2 receptor has quite recently been elucidated (9, 10), this receptor is not coupled to any of the known transduction mechanisms and its biological role remains unclear. At variance with the AT_1 receptor, the binding of AII to the AT_2 subtype is not abolished upon treatment with DTT, a reducing agent for disulfide bridges (1).

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Because pressor effects of AII are mediated by the AT_1 receptor, AT_1-selective nonpeptide antagonists represent potential new therapeutic agents for the treatment of hypertension and have therefore given rise to much interest among pharmaceutical companies. Most of the compounds developed are structurally related to DuP 753; they display a conserved BPT moiety, with their imidazole nucleus being replaced by aromatic nitrogen-containing heterocycles such as imidazo[4,5-*b*]pyridine (L-158,809) (11), aminopyridine (A-81988) (12), triazolinone (SC-51316) (13, 14), quinoline (ICI D8731) (15), *N*-oxide quinoxaline (16), or spirocyclopentaneimidazolinone (SR 47436) (17), whereas CGP 48933 (18) is one of the first non-heterocyclic antagonists. Few compounds either lacking the BPT moiety or displaying a modified BPT group have been synthesized, i.e., SK&F 108566 (19), [[[(1*H*-pyrrol-1-ylacetyl)amino]phenyl]methyl]imidazole derivatives (20), GR117 289 (21), and *N*-phenyl-1*H*-pyrole derivatives (22).

In this context, Fournier Laboratories have synthesized a novel selective nonpeptide AT_1 receptor antagonist, LF 7-0156 (2-[[[2-butyl-1-[(4-carboxyphenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoic acid) (23). Its structure shows that it belongs to the family of compounds that do not have the BPT

ABBREVIATIONS: AII, angiotensin II; DTT, dithiothreitol; BPT, biphenyl tetrazole; CHO, Chinese hamster ovary; Me_2SO, dimethylsulfoxide; BSA, bovine serum albumin; NK, neurokinin; AT_1 receptor, type 1 angiotensin receptor; AT_2 receptor, type 2 angiotensin receptor.

moiety. Therefore, a radiolabeled derivative of LF 7–0156 should provide a new tool for the pharmacological and structural characterization of AT_1 receptors, adding to the list of already described radioactive molecules (24–29); [^{3}H]LF 7–0156 has been synthesized and its binding properties have been studied in several cell systems expressing AT_1 receptors. In addition, a comparison was made with the properties of [^{3}H] DuP 753, the only commercially available nonpeptide radioligand.

Materials and Methods

Chemical Synthesis of LF 7–0156 and [^{3}H]LF 7–0156

LF 7–0156 and its tritiated derivative [^{3}H]LF 7–0156 were synthesized by Fournier Laboratories (Daix, France) (23) and the Commissariat à l'Energie Atomique (CEA, Saclay, France), as summarized in Fig. 1. Tritiation at position 4 on the imidazole ring was achieved by treatment of the chlorinated compound **5** with tritium gas in the presence of palladium on carbon; because this reaction (yield, 25%) was carried out in methanol, exchange reactions between this solvent and tritium are likely to be responsible for the moderate specific radioactivity (4.6 Ci/mmol, measured by UV spectrophotometry and radioactivity determinations). Using dimethylformamide or methyl acetate as solvent at the dechlorination step has allowed production of tritiated analogues displaying expected specific radioactivities (>20 Ci/mmol).[1] The unlabeled derivative LF 7–0156 was synthesized through a similar synthesis scheme, except that dechlorination was carried out on compound **2**; all reaction yields were quite satisfactory (>80%).

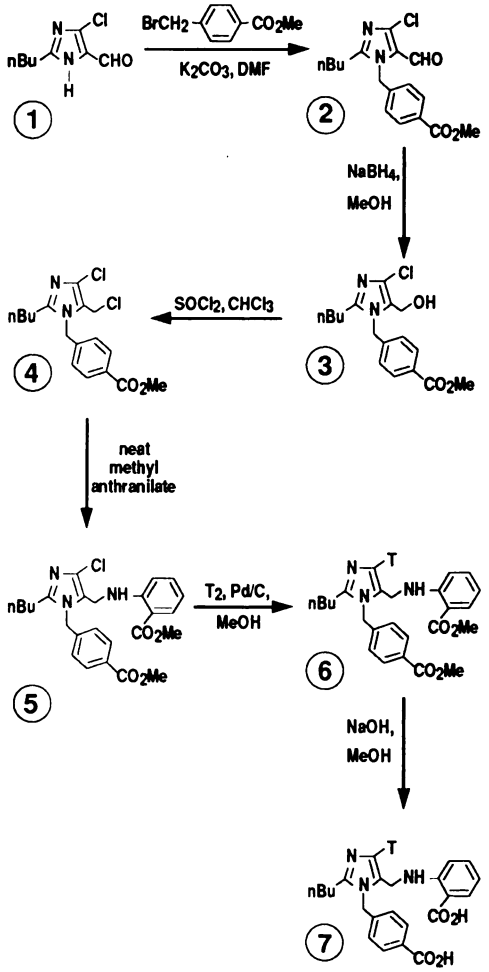

Fig. 1. Chemical synthesis of [^{3}H]LF 7–0156. The yields of the various steps were as follows: **1** to **2**, 90%; **2** to **3**, 98%; **3** to **4**, 95%; **4** to **5**, 90%; **5** to **6**, 25%; **6** to **7**, 83%. Unlabeled LF 7–0156 was synthesized according to a similar scheme, except that dechlorination at the 4-position of the imidazole ring was carried out on compound **2**, with a high yield (89%). *nBu*, *n*-butyl; *Me*, methyl; *DMF*, dimethylformamide; *MeOH*, methanol.

The initial radiochemical purity was 99.4%, as assessed by high performance liquid chromatography analysis. The compound was stored at −20° in a methanol/water (1:1) solution without significant decrease of its purity over a 1-year period of time.

Other Ligands

The other nonpeptide antagonists (DuP 753 and SK&F 108566) were synthesized by Fournier Laboratories. [^{3}H]DuP 753 (specific activity, 40.5–54.9 Ci/mmol) was from New England Nuclear (Mechelen, Belgium). Sar^1-AII was purchased from Bachem (Bubendorf, Switzerland) and radioiodinated (1800 Ci/mmol) as described previously (30).

Functional Assays

Inhibition of AII-induced rabbit aortic ring contraction. New Zealand white rabbits (3.0–3.5 kg) were killed by an intravenous overdose of sodium pentobarbitone. The thoracic aorta was dissected out and transferred to a Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.026 mM EDTA, 5.5 mM glucose), bubbled with 95% O_2/5% CO_2. Eight rings of 4-mm length were prepared from the aortic segment and the endothelium was rubbed off *in vitro* with a pipe cleaner, which was introduced into the vessel lumen and gently moved back and forth several times. Rings were then suspended on triangular stainless-steel wires (0.3-mm diameter) in 20-ml jacketed organ baths maintained at 37°. One hook was suspended from a Gould-Statham UC2 or UTC2 transducer and the other was fixed to a plastic support leg. Changes in isometric tension were recorded continuously with two-channel recorders (Gould BS272 or Linseis type 7025). Rings were left unstretched for 30 min and were then stretched in a stepwise fashion to a passive force of 2 g.

After 1 hr of equilibration, arterial rings were contracted with an isotonic high-KCl (75 mM)-containing solution and the baths were washed three times with normal Krebs solution. This procedure was repeated 30 min later. After return to the base-line, LF 7–0156 was added to the baths at various concentrations, and 15 min later responses to either a single concentration of AII (3 nM) or cumulative additions of AII were obtained. The corresponding IC_{50} and pA_2 values were subsequently calculated (31).

Inositol phosphate accumulation. CHO cells expressing the AT_{1A} receptor were grown in six-well tissue culture clusters and labeled for 2 days with *myo*-[2-^{3}H]inositol (1.5 ml/well of a 2 μCi/ml inositol solution). Before the experiment, the radioactive medium was discarded and the cells were incubated for 1 hr at 37° in culture medium containing neither serum nor tritiated inositol. After a 10-min LiCl pretreatment, the cells were incubated for 15 min at 37° in the presence or absence of receptor ligands, in Mg^{2+}/Ca^{2+}-containing phosphate-buffered saline with 0.1 mg/ml bacitracin. Cell disruption was carried out in 30% perchloric acid; pooled inositol phosphates were isolated by ion exchange chromatography of neutralized extracts on Dowex AG 1-X8 resin (200–400 mesh, formate form), as described previously (32).

CHO Cells

CHO cells expressing the AT_{1A} receptor were kindly supplied by K. E. Bernstein (Atlanta, GA) and E. Clauser (Paris, France).

Membrane Preparations

Rat liver membranes were purified as described previously (30) and were stored frozen in liquid nitrogen before use.

[1] S. Nouet, P. Dodey, P. Renaut, D. Pruneau, R. Larguier, C. Lombard, T. Groblewski, J. Marie, and J. C. Bonnafous, unpublished observations.

Crude ewe lamb uterine membranes were prepared as follows. Ewe lamb uteri (either fresh or stored frozen at −80°) were cut into small pieces and then homogenized in 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, using a Polytron homogenizer (3 × 8 sec, setting 8) and then a Dounce homogenizer. After 10 min of gentle stirring, the homogenate was filtered through one and then two layers of gauze and was centrifuged at 600 × *g* for 20 min. The supernatant was centrifuged at 30,000 × *g* for 20 min. After washing, the membranes were resuspended in homogenizing buffer and frozen in liquid nitrogen.

Crude membranes from CHO cells expressing the AT_{1A} receptor were prepared at 4° as follows. Cells were lysed in ice-cold 10 mM Tris·HCl, pH 7.4, and then homogenized with a Dounce homogenizer. The supernatant of a first 300 × *g* centrifugation was centrifuged at 48,000 × *g* for 20 min. The pellet was resuspended in 10 mM Tris·HCl, pH 7.4, and aliquots of the membrane suspension were frozen in liquid nitrogen.

Protein measurements in membrane preparations were carried out according to the method of Lowry *et al.* (33).

Binding Assays

Liver membranes. Membranes (10 μg/assay and 90-μl volume for ^{125}I-Sar^1-AII binding, 50 μg/assay and 90-μl volume for [^{3}H]DuP 753 binding, and 250 μg/assay and 450-μl volume for [^{3}H]LF 7–0156 binding) were incubated for various periods of time (30 min for equilibrium binding experiments) at 30° in binding medium (50 mM phosphate, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/ml bacitracin, 0.6% Me_2SO) with various amounts of the tested radioligands. Nonspecific binding was determined by addition to the assays of an excess (10^{-5} M final concentration) of either unlabeled Sar^1-AII or unlabeled nonpeptide antagonist (DuP 753 or LF 7–0156). The assays were carried out in triplicate, in polypropylene tubes to minimize the adsorption of nonpeptide ligands. Bound radioactivity was estimated by filtration through GF/C filters (which had been presoaked in a 1% BSA solution), followed by either liquid scintillation or γ counting. The K_i values of the nonpeptide antagonists estimated in competition experiments were calculated according to the method of Ekins *et al.* (34) and applied as described by Korenman (35); the K_i of the tested substances can be related to the dissociation constant (K_0) of the reference substance (unlabeled counterpart of the radioligand) by the equation:

$$K_i = K_0 \cdot \frac{R + 1 - (p/h)}{R(p/h)}$$

where *p* and *h* are the concentrations of reference substance and tested compound, respectively, that produce identical percentages of radioligand binding inhibition and *R* equals [free radioligand]/[receptor-radioligand complex] for this given percentage of inhibition.

Ewe lamb uterus. The AT_1 receptor subtype contained in ewe lamb uterus membranes was inactivated by DTT treatment (20 mM, 1 hr at room temperature). The ability of unlabeled LF 7–0156 to compete with the binding of ^{125}I-Sar^1-AII to the AT_2 receptor (resistant to DTT treatment) was evaluated in 30-μg membrane samples in 90 μl of binding buffer (50 mM Tris·HCl, pH 7.4, 125 mM NaCl, 6.5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 1 mg/ml bacitracin, 0.6% Me_2SO). Estimation of bound radioactivities was performed as described above.

CHO cell membranes. The binding medium was similar to that used for rat liver membranes (no BSA, 0.1 mg/ml bacitracin). The membrane amounts were 10 μg/assay (90-μl volume) and 125 μg/assay (450-μl volume) for the binding of ^{125}I-Sar^1-AII and [^{3}H]LF 7–0156, respectively.

Intact CHO cells. CHO cells expressing the AT_{1A} receptor were grown in F-12 medium supplemented with 10% fetal calf serum (heat-inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml Geneticin. Cells were plated in 12-well tissue culture clusters and grown to confluence (about 5 × 10^5 cells/well). After removal of the culture medium, the cells were washed twice with binding buffer (phosphate-buffered saline, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/ml bacitracin, 0.6% Me_2SO) and then incubated with 300 μl of binding buffer, containing various ligand concentrations, for 4 hr at 4°, with gentle agitation. The reaction was stopped by removal of medium, followed by two rapid washings with ice-cold binding buffer. The cells were collected after addition of 400 μl of 0.1 N NaOH to each well and the associated radioactivity was estimated by either γ or liquid scintillation counting (after neutralization by addition of 100 μl of 0.5 N acetic acid).

Results

Rabbit Aorta Functional Assay

LF 7–0156 did not change the basal tension of the arteries but inhibited AII-induced contraction in a concentration-dependent manner, giving an IC_{50} value of 3.6 ± 0.4 nM (mean ± standard deviation of six experiments). Fig. 2A shows that LF 7–0156 produced a rightward shift of the concentration-contraction curve for AII without affecting the maximal response. Schild regression analysis of the effect of LF 7–0156 on AII gave a pA_2 value of 8.44 ± 0.41, with the slope of the regression line being 1.0 ± 0.1 (Fig. 2B). These results indicate that LF 7–0156 is a potent and competitive antagonist of AII receptor-mediated responses.

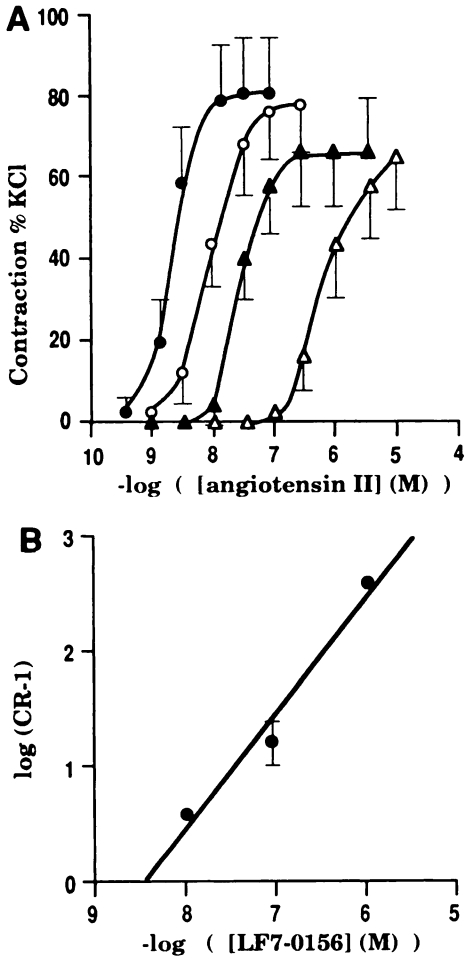

Fig. 2. Effects of LF 7–0156 on contractions of the isolated rabbit aorta in response to AII. A, Cumulative concentration-contraction curves for AII in the absence (●) or presence of 10 (○), 100 (▲), or 1000 nM (△) LF 7–0156. Values represent means ± standard errors of six experiments. B, Graphical display of the antagonism of AII by LF 7–0156, according to Schild regression analysis. The slope of the regression line was 1.0 ± 0.1. The calculated pA_2 value for LF 7–0156 was 8.44 ± 0.41. CR, concentration ratio.

Effect of LF 7-0156 on AII-Induced Inositol Phosphate Production

Saturating amounts of Sar^1-AII (10^{-8} M) were able to induce a 4-fold increase in inositol phosphate accumulation in CHO cells expressing the AT_{1A} receptor. This effect was completely blocked in the presence of 10^{-5} M LF 7-0156 (Table 1), which confirms that the nonpeptide antagonist binds to the AT_1 receptor, which is coupled to phospholipase C.

Inhibition of ^{125}I-Sar^1-AII Binding to the Rat Liver AT_1 Receptor

Binding assays were performed with purified rat liver membranes, which contain only the AT_1 subtype (36), predominantly the AT_{1A} subtype (37). ^{125}I-Sar^1-AII binding experiments were usually carried out in the presence of 1 mg/ml BSA and 1 mg/ml bacitracin to prevent peptide hydrolysis by endogenous proteases and nonspecific binding to membrane proteins. However, nonpeptide antagonists such as DuP 753 and its metabolite Exp 3174 bind nonspecifically to BSA (38). The fact that this trapping was shown to vary considerably with minor chemical modifications of the antagonists (with an extreme value being obtained for DuP 532) (38) emphasizes the difficulty of designing standard assays allowing accurate evaluation of binding parameters.

The ability of our antagonist LF 7-0156 to compete with ^{125}I-Sar^1-AII for liver membrane binding was checked under various BSA and bacitracin concentration conditions. The optimal affinity values deduced from competition curves for the nonpeptide antagonist were obtained in the absence of BSA and in the presence of a limited amount of bacitracin (0.1 mg/ml). Under these conditions, minor hydrolysis of the tracer peptide occurred, as assessed by a slight rightward displacement of competition curves obtained with unlabeled Sar^1-AII in the same experiment. All of the experiments included this competition curve with unlabeled Sar^1-AII; the deduced experimental K_i value for Sar^1-AII (repeatedly found to be 0.6–0.7 nM, instead of 0.3–0.4 nM as found in direct ^{125}I-Sar^1-AII binding experiments) was taken into account in the calculation of the K_i for LF 7-0156, according to the method of Ekins *et al.* (34) applied as described by Korenman (35).

LF 7-0156 (10^{-11} to 10^{-6} M) dose-dependently inhibited the binding of ^{125}I-Sar^1-AII to the rat liver AT_1 receptor; total reversal occurred at a concentration of 10^{-6} M. A K_i value of 12.1 ± 2.9 nM (five experiments) could be calculated from the monophasic curve (Fig. 3). When tested in the same experiment, DuP 753 and SK&F 108566, other selective AT_1 receptor ligands, inhibited the specific binding of ^{125}I-Sar^1-AII with K_i values of 13.0 ± 3.5 nM (three experiments) and 7.2 ± 1.7 nM (three experiments), respectively; these values are consistent with those obtained for both antagonists at AT_1 receptors from various tissues (39, 40).

TABLE 1

Effect of LF 7-0156 on AII-induced inositol phosphate production

CHO cells expressing the AT_{1A} receptor (prelabeled with *myo*-[2-^{3}H]inositol) were incubated in the presence or absence of Sar^1-AII and LF 7-0156, as described in Materials and Methods. Results (mean ± standard deviation of triplicate assays) are expressed as dpm corresponding to inositol phosphate accumulation/cell culture well. They refer to a typical experiment, which was reproduced twice.

	Inositol phosphate accumulation
	dpm/well
Control	2091 ± 22
Sar^1-AII (10^{-8} M)	8401 ± 360
Sar^1-AII (10^{-8} M) + LF 7-0156 (10^{-5} M)	1975 ± 158

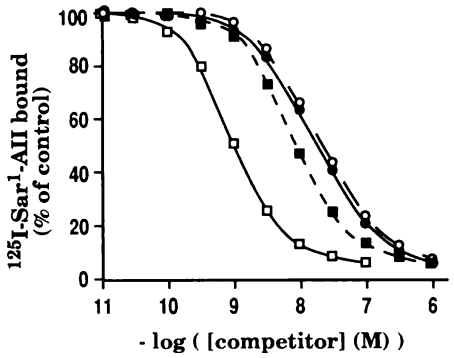

Fig. 3. Inhibition by nonpeptide antagonists of ^{125}I-Sar^1-AII binding to purified rat liver membranes. Membrane samples were incubated in triplicate with ^{125}I-Sar^1-AII (0.2 nM), in the presence of various concentrations of Sar^1-AII (□) (reference curve), LF 7-0156 (●), SK&F 108566 (■), or DuP 753 (○), as described in Materials and Methods. The calculated K_i values (see Materials and Methods) for this typical experiment were 14.3 nM, 6.0 nM, and 15.5 nM for LF 7-0156, SK&F 108566, and DuP 753, respectively. The data are representative of five separate experiments for LF 7-0156 (K_i = 12.1 ± 2.9 nM) and three separate experiments for DuP 753 (K_i = 13.0 ± 3.5 nM) and SK&F 108566 (K_i = 7.2 ± 1.7 nM).

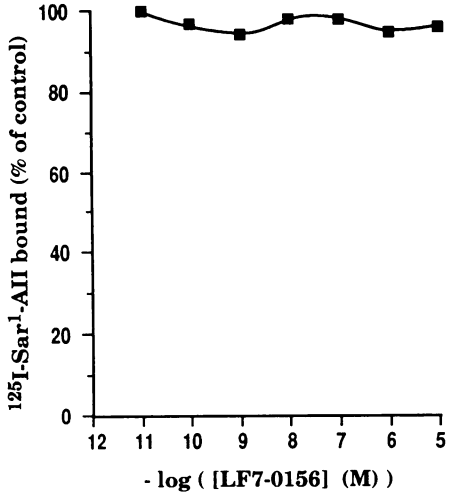

Fig. 4. Effect of LF 7-0156 on ^{125}I-Sar^1-AII binding to ewe lamb uterus membranes. Samples of DTT-treated membranes were incubated in triplicate with ^{125}I-Sar^1-AII (0.4 nM) in the presence of various concentrations of LF 7-0156, as described in Materials and Methods. The presented typical experiment was reproduced twice, with no significant inhibition of radiolabeled peptide binding.

Specificity of LF 7-0156 for the AT_1 Subtype

LF 7-0156 is a potent antagonist of the vascular AT_1 receptor and inhibitor of ^{125}I-Sar^1-AII binding to rat liver membranes. To assess its specificity for the AT_1 subtype, we checked its ability to inhibit ^{125}I-Sar^1-AII binding to the AT_2 receptor. The experiments were performed on ewe lamb uterus membranes (which contain both AT_1 and AT_2 receptors) that had been previously treated with DTT to selectively abolish the binding of AII to the AT_1 subtype (1). When tested at concentrations up to 10^{-5} M, LF 7-0156 did not significantly reduce the binding of ^{125}I-Sar^1-AII to the AT_2 receptor from ewe lamb membranes (the K_d of ^{125}I-Sar^1-AII for this receptor was 0.4 nM; data not shown) (Fig. 4).

Association and Dissociation Kinetics of [^{3}H]LF 7–0156 Binding to Rat Liver Membranes

Association of [^{3}H]LF 7–0156 (Fig. 5) to rat liver membranes revealed that equilibrium was reached with 20–30-min incubation times; the dissociation of bound radioactive ligand produced by an excess of unlabeled compound was rapid, characterized by a k_{-1} value of 0.126 min^{-1}. The k_1 value subsequently calculated from the association curve was 0.012 nM^{-1} min^{-1}. The resulting k_{-1}/k_1 ratio (10.5 nM) is in agreement with K_i values deduced from equilibrium binding experiments (12.1 nM for competition binding, 15.7 nM for direct radioligand binding experiments).

Binding of [^{3}H]LF 7–0156 to Rat Liver Membranes and Comparison with [^{3}H]DuP 753

The binding of [^{3}H]LF 7–0156 was studied with purified rat liver membranes. The careful evaluation of nonspecific binding required comparison of two types of assays, carried out in the presence of an excess of either unlabeled AII or unlabeled nonpeptide ligand itself. The binding properties of [^{3}H]LF 7–0156 were compared, in the same experiment, with those of [^{3}H]DuP 753, which displays significantly different structural features and has been characterized previously (24).

Estimation of binding properties. [^{3}H]LF 7–0156 showed saturable and reversible binding to rat liver membranes. Binding parameters deduced from Scatchard analysis were independent of the excess unlabeled ligand used to evaluate nonspecific binding [K_d values of 12.6 ± 3.1 and 15.7 ± 4.8 nM and B_{max} values of 1.4 ± 0.8 and 1.8 ± 0.4 pmol/mg of protein with excess Sar^1-AII and LF 7–0156, respectively; three experiments] (Fig. 6C). A minor distortion in the linearity of the curve was observed only for high radioligand concentrations. The B_{max} value was consistent with that determined with a saturating amount of ^{125}I-Sar^1-AII (41). Nonspecific binding appeared quite acceptable (Fig. 6A); it represented only 21% of total binding for a ligand concentration of 75 nM, when measured in the presence of an excess of Sar^1-AII.

In contrast, the analysis of [^{3}H]DuP 753 binding to liver membranes, carried out in the same experiment, revealed that this nonpeptide antagonist interacts with a large number of binding sites different from AII receptors; indeed, whereas

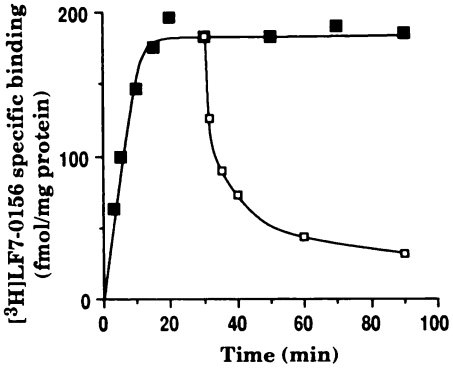

Fig. 5. Association and dissociation kinetics of [^{3}H]LF 7–0156 binding to rat liver membranes. Rat liver membranes were incubated in the presence of 2.4 nM [^{3}H]LF 7–0156, as described in Materials and Methods, for various periods of time. After 30 min, an excess (8.5 μM) of unlabeled LF 7–0156 was added and the decrease in radioligand binding was evaluated. Calculation of k_{-1} and k_1 (k_{-1} = 0.126 min^{-1}, k_1 = 0.012 nM^{-1} min^{-1}) allowed estimation of the dissociation constant (K_d = 10.5 nM). ■, association curve; □, dissociation curve.

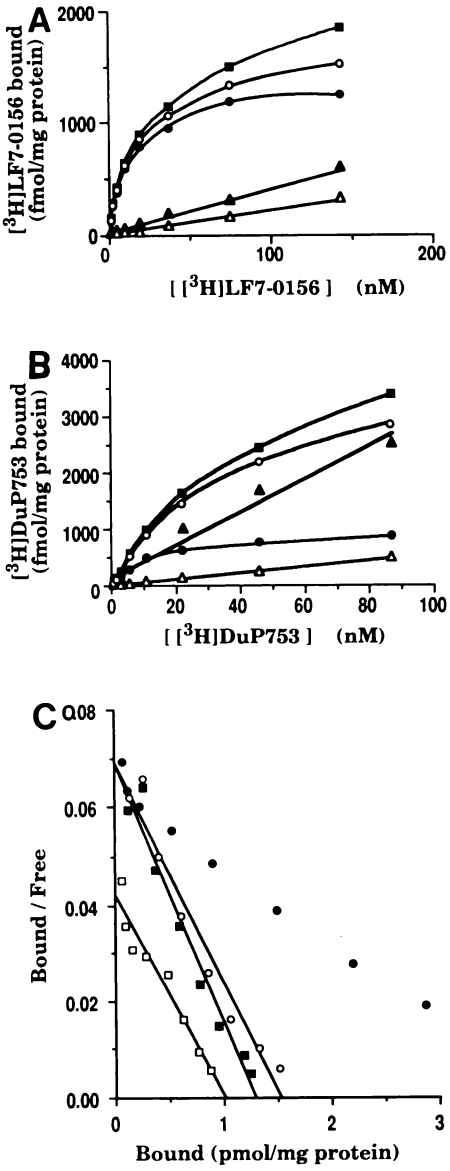

Fig. 6. Binding properties of [^{3}H]LF 7–0156 and [^{3}H]DuP 753 with purified rat liver membranes. The binding of [^{3}H]LF 7–0156 and [^{3}H]DuP 753 to rat liver membranes was carried out as described in Materials and Methods, with the nonspecific binding of both antagonists being estimated in the presence of an excess of either unlabeled antagonist itself or unlabeled Sar^1-AII. A and B, Saturation curves relative to [^{3}H]LF 7–0156 (A) and [^{3}H]DuP 753 (B) binding. ■, Total binding; ▲ and △, nonspecific binding measured in the presence of an excess of either Sar^1-AII or unlabeled nonpeptide antagonist, respectively; ● and ○, specific binding calculated under these two conditions. C, Scatchard analysis of the data corresponding to a typical experiment. Binding of [^{3}H]LF 7–0156 was reversed by LF 7–0156 (○) (K_d = 10.4 nM, B_{max} = 1.5 pmol/mg) or Sar^1-AII (■) (K_d = 10.4 nM, B_{max} = 1.4 pmol/mg of protein) and binding of [^{3}H]DuP 753 was reversed by DuP 753 (●) or Sar^1-AII (□). The indicated values are the means of triplicate assays. The presented data are representative of three separate experiments, which gave similar results (binding of [^{3}H]LF 7–0156 reversed by LF 7–0156, K_d = 15.7 ± 4.8 nM, B_{max} = 1.8 ± 0.4 pmol/mg of protein; binding of [^{3}H]LF 7–0156 reversed by Sar^1-AII, K_d = 12.6 ± 3.1 nM, B_{max} = 1.4 ± 0.8 pmol/mg of protein; binding of [^{3}H]DuP 753 reversed by Sar^1-AII, K_d = 13.8 ± 0.8 nM, B_{max} = 888 ± 126 fmol/mg of protein).

linear Scatchard plots (Fig. 6C) were obtained when nonspecific binding was measured with an excess of Sar[1]-AII (K_d = 13.8 ± 0.8 nM, B_{max} = 888 ± 126 fmol/mg of protein, three experiments), no saturation could be observed when excess unlabeled DuP 753 was used. The values corresponding to both types of assays (Fig. 6B) clearly demonstrate that liver membranes display a high level of "pseudospecific" binding of DuP 753, which is unrelated to AII receptor sites; when measured in the presence of an excess of Sar[1]-AII, the nonspecific binding of [^{3}H]DuP 753 reached 51% and 84% of total binding for 11 nM and 87 nM ligand concentrations, respectively, whereas it was limited to 7% and 15% when an excess of DuP 753 was used.

For unexplained reasons, the B_{max} value for [^{3}H]DuP 753 binding to AII receptor sites was systematically found to be lower than the B_{max} value for [^{3}H]LF 7–0156; we have ruled out the possibility that this could result from inaccurate estimations of the specific radioactivities of the ligands.

These binding studies carried out on highly purified rat liver membranes demonstrate that [^{3}H]LF 7–0156 is a convenient nonpeptide ligand specific for the AT_1 receptor; its nonspecific binding is much lower than that obtained with [^{3}H]DuP 753, which binds pseudospecifically to a large number of membrane sites other than angiotensin receptors.

Relationships between DuP 753 and LF 7–0156 binding sites. Because DuP 753 and LF 7–0156 present some structural differences, it appeared interesting to determine whether their binding sites are superimposable. The elevated pseudospecific binding of [^{3}H]DuP 753 to membranes would make it difficult to interpret competition with its binding by unlabeled LF 7–0156; thus, we performed competition experiments with [^{3}H]LF 7–0156 and unlabeled DuP 753. DuP 753 was able to inhibit the specific binding of [^{3}H]LF 7–0156 to the rat liver receptor in a dose-dependent and monophasic manner (Fig. 7); only 10% of the radiolabeled antagonist was not displaced by the highest DuP 753 concentration (3×10^{-6} M).

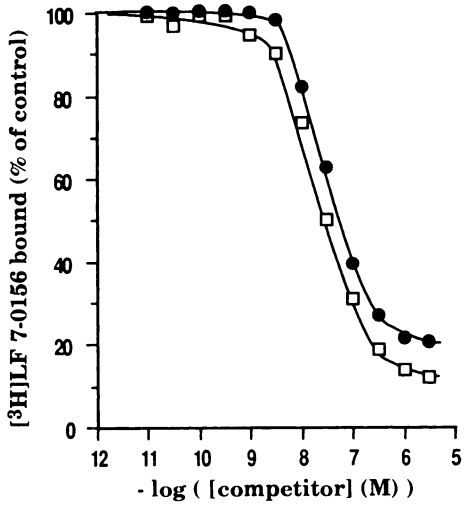

Fig. 7. Inhibition by LF 7–0156 and DuP 753 of [^{3}H]LF 7–0156 binding to purified rat liver membranes. Membranes were incubated with [^{3}H]LF 7–0156 (13.5 nM) and various concentrations of LF 7–0156 (□) (reference curve) or DuP 753 (●), as described in Materials and Methods. The calculated K_i value [according to the method of Ekins *et al.* (34, 35)] for [^{3}H]LF 7–0156 binding inhibition by DuP 753 was 35 nM. The presented data refer to a typical experiment, which is representative of three separate experiments (K_d = 30.8 ± 5.9 nM) The assays were carried out in triplicate.

The calculated K_i value relative to DuP 753 inhibition of [^{3}H]LF 7–0156 binding was systematically found to be higher (30.8 ± 5.9 nM, three experiments) than that found for DuP 753 inhibition of ^{125}I-Sar[1]-AII binding. Although the difference is moderate, it suggests that LF 7–0156 and Sar[1]-AII binding sites might not be fully superimposed and that the two nonpeptide antagonists might display differences in their interactions with the AT_1 receptor.

Effect of reducing or alkylating agents on [^{3}H]LF 7–0156 binding to the AT_1 receptor. Because AII binding to the AT_1 receptor was suppressed upon reduction of essential disulfide bridges (1), it was interesting to determine whether the same treatment affected nonpeptide antagonist binding; DTT pretreatment of the membranes greatly affected [^{3}H]LF 7–0156 binding, although some residual specific binding was observed at the tested ligand concentrations (Table 2). Widdowson *et al.* (42) and Chansel *et al.* (43) also found that the binding of [^{3}H]DuP 753 to rat liver AII-specific binding sites was inhibited by DTT treatment.

Moreover, cysteine alkylation by *N*-ethylmaleimide was found to decrease Sar[1]-AII binding to the rat liver receptor (Table 2). Here again, [^{3}H]LF 7–0156 recognition by the alkylated receptor was affected, compared with the control receptor (Table 2). Consequently, the tested chemical treatments of the receptor did not allow a striking discrimination between peptide and our nonpeptide radioligand.

Binding of [^{3}H]LF 7–0156 to the AT_{1A} Receptor Expressed in CHO Cells

As a prerequisite for future topographical studies involving analysis of ligand binding to mutated receptors, we have determined the optimal conditions for [^{3}H]LF 7–0156 binding to the AT_{1A} receptor stably expressed in CHO cells.

Binding to membrane preparations. As with the rat liver AT_1 receptor, [^{3}H]LF 7–0156 showed saturable and reversible binding to crude membranes from CHO cells expressing the AT_1 subtype. The data were characterized by linear Scatchard plots and the binding parameters were independent of the method of evaluating nonspecific binding (saturation curves in Fig. 8A), using an excess of either unlabeled Sar[1]-AII (K_d = 7.5 ± 1.1 nM, B_{max} = 2.0 ± 0.3 pmol/mg of protein, three experiments) or unlabeled LF 7–0156 (K_d = 7.9 ± 1.5 nM, B_{max} = 2.1

TABLE 2

Effects of DTT and *N*-ethylmaleimide on agonist and nonpeptide antagonist binding to rat liver AII receptors

Rat liver membranes were treated for 1 hr with DTT or for 30 min with N-ethylmaleimide (NEM) at room temperature (pH 7.8) before radioligand binding evaluation, as described in Materials and Methods. Data are expressed as the percentage of binding observed for control membranes. They refer to a typical experiment, which was repeated three times with similar results. The values represent the means of triplicate experiments, with standard deviations of <10%.

Radioligand	Residual specific binding after membrane treatment			
	DTT, 20 mM	NEM		
		3 mM	10 mM	30 mM
	% of control			
^{125}I-Sar[1]-AII				
0.5 nM	1	56	21	4
4 nM	6	75	40	13
[^{3}H]LF 7-0156				
11 nM	16	89	33	9
75 nM	26	100	55	24

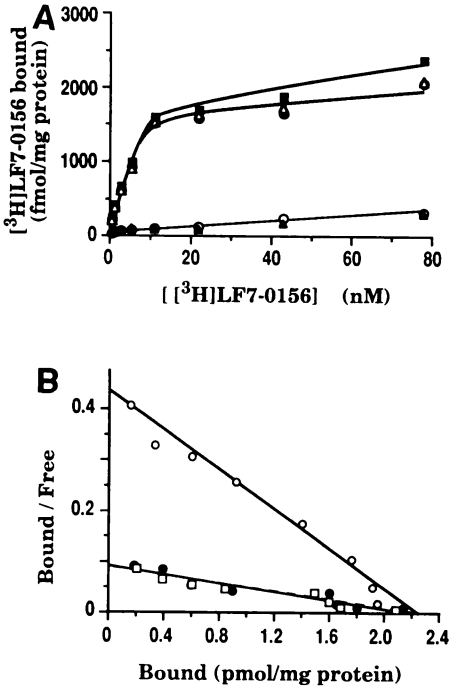

Fig. 8. Binding properties of ^{125}I-Sar^1-AII and [^{3}H]LF 7–0156 with crude membranes from CHO cells expressing the AT_{1A} receptor. The binding of ^{125}I-Sar^1-AII and [^{3}H]LF 7–0156 to membranes from CHO cells was performed as described in Materials and Methods, with the nonspecific binding of [^{3}H]LF 7–0156 being determined in the presence of an excess of either unlabeled antagonist itself or unlabeled Sar^1-AII. A, Saturation curves, relative to [^{3}H]LF 7–0156 binding. ■, Total binding; ○ and ▲, nonspecific binding measured in the presence of an excess of either Sar^1-AII or unlabeled LF 7–0156, respectively; ● and △, specific binding calculated under the two experimental conditions. B, Scatchard analysis of data corresponding to a typical experiment, measuring binding of ^{125}I-Sar^1-AII (○) (K_d = 0.6 nM, B_{max} = 2.3 pmol/mg of protein) or binding of [^{3}H]LF 7–0156 reversed by LF 7–0156 (●) (K_d = 6.3 nM, B_{max} = 2.2 pmol/mg of protein) or Sar^1-AII (□) (K_d = 6.7 nM, B_{max} = 2.2 pmol/mg of protein). The indicated values are the means of triplicate assays and are representative of three separate experiments, which gave similar results ([^{3}H]LF 7–0156 binding reversed by LF 7–0156, K_d = 7.9 ± 1.5 nM, B_{max} = 2.1 ± 0.4 pmol/mg of protein; [^{3}H]LF 7–0156 binding reversed by Sar^1-AII, K_d = 7.5 ± 1.1 nM, B_{max} = 2.0 ± 0.3 pmol/mg of protein).

± 0.4 pmol/mg of protein) (Fig. 8B). The binding capacity was similar to that found for ^{125}I-Sar^1-AII with the same membrane preparations. Apart from a better affinity of our tritiated nonpeptide antagonist for the CHO cell receptor than for the rat liver receptor, the nonspecific binding of the antagonist appeared to be reduced in CHO cell membranes (Fig. 8A).

Binding to intact cells. The purpose of this study was to evaluate the affinity of [^{3}H]LF 7–0156 for the AT_{1A} receptor, under conditions where cell disruption and membrane preparation have been avoided. The binding assays with intact cells were performed at 4°, so that a 4-hr incubation period was required to reach equilibrium. The binding properties of [^{3}H]LF 7–0156 with intact cells were similar to those observed for membrane preparations, with a slightly improved affinity (K_d = 3.5 ± 0.5 nM, B_{max} = 1.2 ± 0.1 pmol/10^6 cells, mean of four experiments) and still lower nonspecific binding (Fig. 9).

Discussion

LF 7–0156 is a new nonpeptide AII antagonist, designed by Fournier Laboratories (23); its ability to inhibit AII-induced

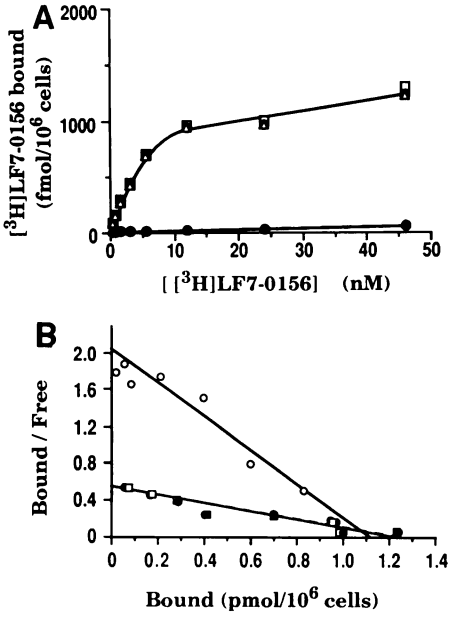

Fig. 9. Binding properties of ^{125}I-Sar^1-AII and [^{3}H]LF 7–0156 with intact CHO cells expressing the AT_{1A} receptor. The binding of ^{125}I-Sar^1-AII and [^{3}H]LF 7–0156 to intact CHO cells expressing the AT_{1A} receptor was carried out as described in Materials and Methods, with the nonspecific binding of [^{3}H]LF 7–0156 being estimated in the presence of an excess of either unlabeled antagonist itself or unlabeled Sar^1-AII. A, Saturation curves, relative to [^{3}H]LF 7–0156 binding. □, Total binding; ○ and ▲, nonspecific binding measured in the presence of an excess of either Sar^1-AII or unlabeled LF 7–0156, respectively; ● and △, specific binding calculated under the two experimental conditions. B, Scatchard analysis of the data corresponding to a typical experiment, measuring binding of ^{125}I-Sar^1-AII (○) (K_d = 0.9 nM, B_{max} = 1.1 pmol/10^6 cells) or binding of [^{3}H] LF 7–0156 reversed by LF 7–0156 (●) (K_d = 3.9 nM, B_{max} = 1.2 pmol/10^6 cells) or Sar^1-AII (□) (K_d = 3.9 nM, B_{max} = 1.2 pmol/10^6 cells). The indicated values are the means of triplicate assays and are representative of four separate experiments, which gave identical results (K_d = 3.5 ± 0.5 nM, B_{max} = 1.2 ± 0.1 pmol/10^6 cells).

contraction of rabbit aortic strips *in vitro* was characterized by an IC_{50} value of 3.6 nM. Moreover, it is devoid of any agonistic activity. In the present study, we used LF 7–0156 as a novel tool for the pharmacological characterization of the AT_1 receptor and for future mapping studies. This led us to synthesize the tritiated compound [^{3}H]LF 7–0156 and evaluate its binding properties with several membrane or cell preparations.

Initial experiments showed that LF 7–0156 was a potent competitor of the binding of ^{125}I-Sar^1-AII to rat liver membranes, which contain only the AT_1 receptor, predominantly the AT_{1A} subtype (36, 37); although lower than that of SK&F 108566 (K_i = 7.2 nM), its affinity (K_i = 12.1 nM) was found to be similar to that of DuP 753 (K_i = 13.0 nM), with the binding parameters of the reference nonpeptide antagonists being consistent with previously published data (39, 40). The specificity for the AT_1 subtype was assessed from the inability of LF 7–0156 to recognize the AT_2 receptor from ewe lamb uterus.

The binding of [^{3}H]LF 7–0156 to purified rat liver membrane preparations revealed a single class of high affinity sites; the K_d value (12.6 nM) was in close agreement with that deduced from competition experiments. The reversal of this binding by an excess of either LF 7–0156 or Sar^1-AII demonstrated that only AII receptor sites were labeled by the nonpeptide antagonist.

Our analysis also included the study of the properties of [^{3}H]

DuP 753. In addition to its binding to specific AII sites (K_d = 13.8 nM), we found evidence for an important pseudospecific binding of [^{3}H]DuP 753 to rat liver membranes; this binding could hardly reach a saturation level and is obviously irrelevant to receptor recognition. This problem of pseudospecific binding was not reported in the initial work describing the properties of [^{3}H]DuP 753 (24) or the parent radioiodinated compound ^{125}I-Exp 985 (25). However, the fact that [^{3}H]DuP 753 can bind to non-angiotensin binding sites in rat liver has been well documented by Widdowson *et al.* (42) and Chansel *et al.* (43). The same phenomenon was observed for the binding of [^{3}H]L-158,809 (Merck Research Laboratories) to rat kidney membranes and, to a lesser extent, to rat liver membranes (26). The subnanomolar K_d values for this compound and for the Abbott compound [^{3}H]A-81988 (27), which allow experiments to be performed using lower concentration ranges, probably make pseudospecific binding acceptable. The absence of the BPT moiety in [^{3}H]LF 7–0156 can be postulated to be responsible for its higher binding specificity, despite its significantly lower affinity.

This unique structure, together with the properties described in the present paper, make [^{3}H]LF 7–0156 an interesting pharmacological tool. An important question concerns the comparison of epitopes involved in the interaction of the AT_1 receptor with angiotensin peptides and nonpeptide antagonists. Recent studies on NK receptors (44, 45) or cholecystokinin-B/gastrin receptors (46) emphasized discrimination between domains involved in peptide and nonpeptide ligand recognition. Moreover, subtle changes in the structure of the receptors, afforded by interspecies differences, have been shown to affect the specificity for nonpeptide antagonists (46, 47). Quite recently, Gether *et al.* (48) proposed that two nonpeptide tachykinin antagonists act through epitopes located on corresponding segments of NK1 and NK2 receptors.

Concerning AT_1 receptor antagonists, an important issue is the extent of spatial overlap between the active conformations of structurally different molecules, i.e., DuP 753 or L-158,809, compared with SK&F 108566 or LF 7–0156, which do not possess the BPT moiety. Our preliminary competition experiments with LF 7–0156 and DuP 753 indicate a noncomplete coincidence of the binding sites for the two antagonists. It is noteworthy that different modes of superimposition with AII itself have been proposed for DuP 753-related compounds (49) and SK&F 108566-related compounds (50).

Obviously, genetic engineering of the AT_1 receptor will allow a better understanding, at the molecular level, of the topography of its interaction with peptide ligands and various nonpeptide antagonists. In this respect the radiolabeled antagonist [^{3}H]LF 7–0156 should constitute an appropriate tool, inasmuch as its binding to intact CHO cells expressing the AT_{1A} receptor subtype is characterized by high affinity (K_d = 3.5 nM) and a total absence of nonspecific binding. It will facilitate experimentation on mutated receptors expressed in this cell system, permitting accurate analysis of receptors possibly displaying greatly decreased affinity, and will allow dissection of possible differences in receptor amino acids essential for the recognition of peptide or various nonpeptide ligands. Such differences are exemplified by the recent cloning of *Xenopus laevis* heart (51, 52) and turkey adrenal (53) receptors, which have no affinity or strongly reduced affinity for nonpeptide antagonists. A complementary biochemical approach for the analysis of the nonpeptide binding site would consist of the covalent labeling of the receptor with appropriate photoactivable probes. The lack of nonspecific binding of [^{3}H]LF 7–0156, together with the presence of the anthranilate moiety in the imidazole 5-position chain of LF 7–0156, has allowed the synthesis of a closely related compound suitable for AT_1 receptor photolabeling.[2] Comparison of receptor photolabeling patterns using azido peptidic (41) or nonpeptidic probes should be informative for a comparative analysis of their binding sites.

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