Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 209-217

DuP 532 : A SECOND GENERATION OF NONPEPTIDE ANGIOTENSIN II RECEPTOR ANTAGONISTS

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Received April 8, 1991

SUMMARY. DuP 532 is a novel nonpeptide angiotensin II (AII) receptor antagonist under development for the treatment of hypertension. DuP 532 is a more potent antihypertensive agent in renal hypertensive rats (ED_{30} = 0.042 mg/kg, i.v.) and displays a similar or longer duration of action than the previously described AII antagonist, DuP 753. DuP 532, in contrast to DuP 753, is a noncompetitive antagonist of AII-induced contractions of rabbit aortic strips (K_B = 1.1 x 10^{-10}M). However, the inhibition of AII binding by DuP 532 in rat adrenal cortex does not correlate with either the aortic contractile response or with the hypotensive response. Assay conditions were evaluated and the presence or absence of BSA was shown to markedly affect the apparent binding affinity of DuP 532 and other 5-carboxylic acid derivatives. DuP 753 and other compounds were much less affected. The IC_{50} for DuP 532 was 4.7 x 10^{-6}M with and 3 x 10^{-9}M without BSA. The IC_{50}s for DuP 753 were 1.7 x 10^{-8}M with and 5 x $^{-9}$M without BSA. Both compounds with or without BSA did not completely inhibit AII binding which is characteristic of AT_1 selectivity. BSA also reduced the effect of DuP 532 on the AII-induced contractions of rat main pulmonary artery preparations and the AII-induced Ca^{2+} mobilization in rat aortic smooth muscle cells. DuP 532 was very specific for AT_1 receptors and did not interfere with receptors associated with neurotensin, prazosin, bradykinin, nitrendipine, or vasopressin. It is concluded that DuP 532 represents a new class of specific, but noncompetitive, AII receptor antagonists whose binding characteristics may provide new insight into AII receptor function.

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0006-291X/91 $1.50

DuP 753 and its forebearing congeners (1,2) may all be classified as the first generation of nonpeptide angiotensin II (AII) blockers which show specificity and high affinity for the type-1 angiotensin receptors (AT_1). This class of compounds is distinguished by its competitive mode of action and is devoid of partial agonist activity. Both of these characteristics are in sharp contrast with that of peptide antagonists such as saralasin and [Sar^1Ile^8]AII (2,3). DuP 753 exhibits a rapid onset of action and high dissociability (4). It inhibits the AII dose-responses, both in vitro and in vivo, by shifting the curves to the right without any diminuation of the maximal response (2,5).

The development of DuP 753 and more potent AII antagonists (1) had provided some unusual structure-affinity-function relationships. It became clear that compounds with a free carboxylic acids in the 5-position of the imidazole ring were very potent and conferred noncompetitive antagonistic properties. DuP 532 is the prototype of this subclass of biphenyltetrazole chosen for clinical testing because of its excellent antihypertensive potency and duration of action. What was puzzling was the discrepancy between the high potency in vivo and relatively low potency in the binding assay. The present report describes the receptor binding characteristics of DuP 532 and the results of experiments designed to resolve this discrepancy.

MATERIALS AND METHODS

Drugs - DuP 532, 2-propyl-4-pentafluoroethyl-1-[(2'-(1**H**-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid; EXP3174, 2-n-butyl-4-chloro-1-[(2'-(1**H**-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole -5-carboxylic acid; EXP3892, 2-n-propyl-4-trifluoromethyl-1-[(2'-(1**H**-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid and DuP 753 were synthesized at E.I. du Pont de Nemours and Company (Wilmington, DE). Saralasin and AII were purchased from Sigma Chemical Co. (St. Louis, MO) whereas [^{125}I]AII and ^{14}C-DuP 532 (S.A. 38.93 mCi/mmol) were obtained from DuPont-New England Nuclear Products (Boston, MA). [Biphenyl-3-^{3}H] EXP3174 (S.A. 9.68 Ci/mmol) was synthesized at Merck Sharp and Dohme (Rahway, N.J.). All nonpeptide AII antagonists were dissolved either in DMSO or 5% $NaHCO_3$ and further diluted in the appropriate buffers.

Ligand-receptor Binding Studies - Rat adrenal microsomes were prepared according to procedures described by Chiu et al. (2). Receptor bindings were performed by incubating aliquots of the microsomes with 0.05 nM [^{125}I]AII and varying concentrations of inhibitor in a final volume of 0.5 ml of assay buffer (pH 7.2) containing 5 mM $MgCl_2$, 50 mM Tris, and w/wo 0.25% bovine serum albumin (BSA). After 60 min of incubation at 25° C, the bound radioactivity were separated and counted in a gamma counter.

Contractile Studies - Main pulmonary arteries were dissected from Sprague-Dawley rats (300-375 gms) were mounted in tissue baths for recording of contraction as described by Chand and Altura (6). Cumulative concentration-response curves to AII were determined alone and in the same tissues that had been incubated for 1 hour with vehicle or various concentrations of DuP 532. The experiments were conducted in a similar manner using Krebs buffer with or without 0.2% BSA at 37° C.

Ca^{2+} Mobilization assay - Changes of free cytoplasmic Ca^{2+} was monitored as described by Chiu et al. (7). Fura-2 loaded rat aortic smooth muscle cells were suspended in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, w/wo 0.5 mg/mL BSA, 20 mM Hepes buffer, pH 7.4). AII-induced increases in Ca^{2+} fluorescence at 25° C was expressed as 340/380 ratios after background subtraction. The effect of an antagonist was presented as a percent of the effective change induced by 3 x 10^{-8}M AII.

Binding to BSA- [^{14}C]DuP 532 , [^{3}H]DuP 753 or [^{125}I]AII was incubated in the binding assay buffer with or without 0.25% BSA for 5 or 60 min at 25° C. The samples were filtered through Millipore Ultrafilters (Bedford, MA) that retain molecules greater than 10,000 dalton. The filters were washed once with 1 mL of cold buffer. The radioactivity retained in the filters and the filtrates were assessed by scintillation counting.

RESULTS

Correlation between binding and functional antagonism. The inhibitory binding constants (K_i's) for a series of nonpeptide AII antagonists were obtained from the inhibition of [^{3}H] or [^{125}I]AII binding in rat adrenal cortical microsomes (Fig. 1). Functional antagonism (pA_2) of the AII-induced contraction was assessed on rabbit aortic strips (2). As shown in figure 1A, a highly significant correlation exists between the K_i and the pA_2 values for the majority of nonpeptide AII antagonists except for an unique group of compounds typified by DuP 532, EXP3174 and EXP3892. A similar phenomenon was observed when correlating the K_i values with the antihypertensive potencies in conscious renal artery-ligated hypertensive rats (Fig. 1B). The results indicate that the inhibitory potencies for this series of compounds are much greater than predicted from their apparent receptor affinities.

The influence of BSA on ligand-receptor binding. [^{125}I]AII binding to rat adrenal cortical microsomes was evaluated in the presence or absence of 0.25% BSA. Figure 2A illustrates that the inhibition of specifically bound [^{125}I]AII by unlabeled AII (3 x 10^{-10} to 10^{-8}M) was unaffected by the absence of BSA. Identical IC_{50}s (4 x 10^{-9}M) were obtained in both conditions. The concentration-inhibition curve for DuP 753 was slightly shifted to the left in the absence of BSA increasing the apparent affinity about three-fold (IC_{50}: 1.7 x 10^{-8}M w/BSA vs 5 x 10^{-9}M

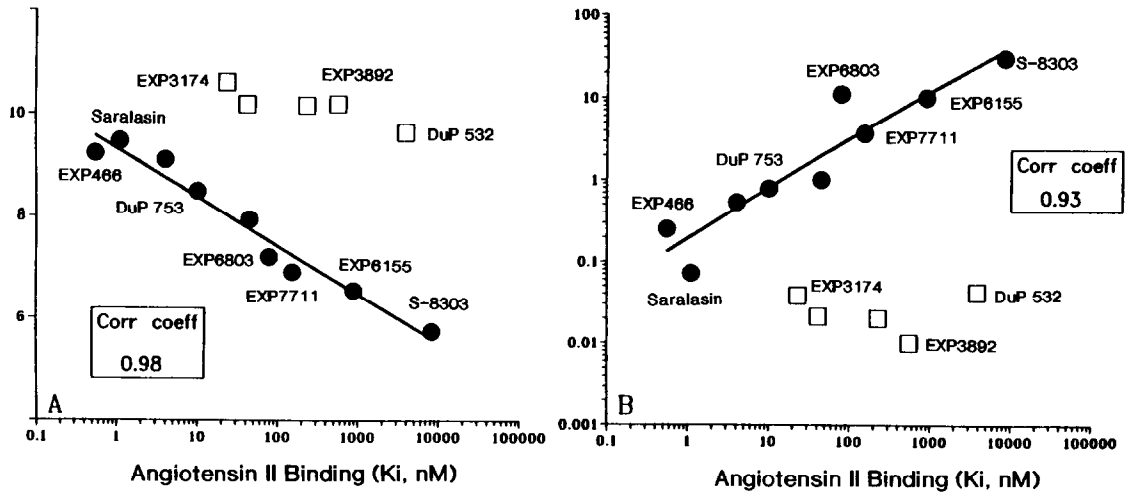


Fig. 1. Correlation between AT_1 receptor affinities (k_is) from rat adrenal cortical microsomes and functional AII antagonistic potencies in rabbit aorta (pA_2s, panel A) and between K_is and antihypertensive potencies (IV ED_{30}, panel B) in renal-hypertensive rats of a series of nonpeptide AII antagonists (see structures in ref. 4,8).

w/o BSA) (Fig. 2B). EXP3174 was also shifted to the left with an increase of 20-fold (Fig. 2C). Finally, a 1500-fold shift to the left was observed with DuP 532 when BSA was absent, yielding an IC_{50} value of 3 x 10^{-9}M (Fig. 2D). As a result the correlation between inhibitory binding constants obtained in the absence of BSA and the pA_2 values thus becomes highly significant (corr coeff 0.85).

The effect of BSA on AII-induced contraction. In the rat main pulmonary artery addition of BSA (0.2%) to the bathing buffer did not significantly alter the AII concentration-contraction responses. As shown in Figure 3, in the absence of BSA, DuP 532 (0.067 and 0.22 nM) produced a shift to the right in the concentration-response curve to AII which was associated with a reduction in the maximal contractile response. The suppression of the AII maximal response amounted to 31±15% and 85±2% at the low and high concentration of DuP 532, respectively. However, the presence of BSA markedly reduced the ability of DuP 532 to attenuate AII responses. As a result, concentrations of DuP 532 as high as 22 nM produced only a 36±12% reduction in the maximal response and a rightward shift of the AII curve in a magnitude equivalent to that produced by a concentration of 0.067 nM in the absence of BSA.

The effect of BSA on AII-induced Ca^{2+} mobilization in rat aortic smooth muscle cells. At 3 x 10^{-8}M, AII elicited 80% of the maximal increase in Ca^{2+} fluorescence (7). This response was not altered when (0.05%) BSA was absent in the incubation buffer (data not shown). With the BSA present, DuP 532 (10^{-7} to 10^{-5}M) inhibited the AII-induced response in a concentration-dependent manner yielding an IC_{50} value of 1.2 x 10^{-6}M. In contrast, in the absence of BSA, the

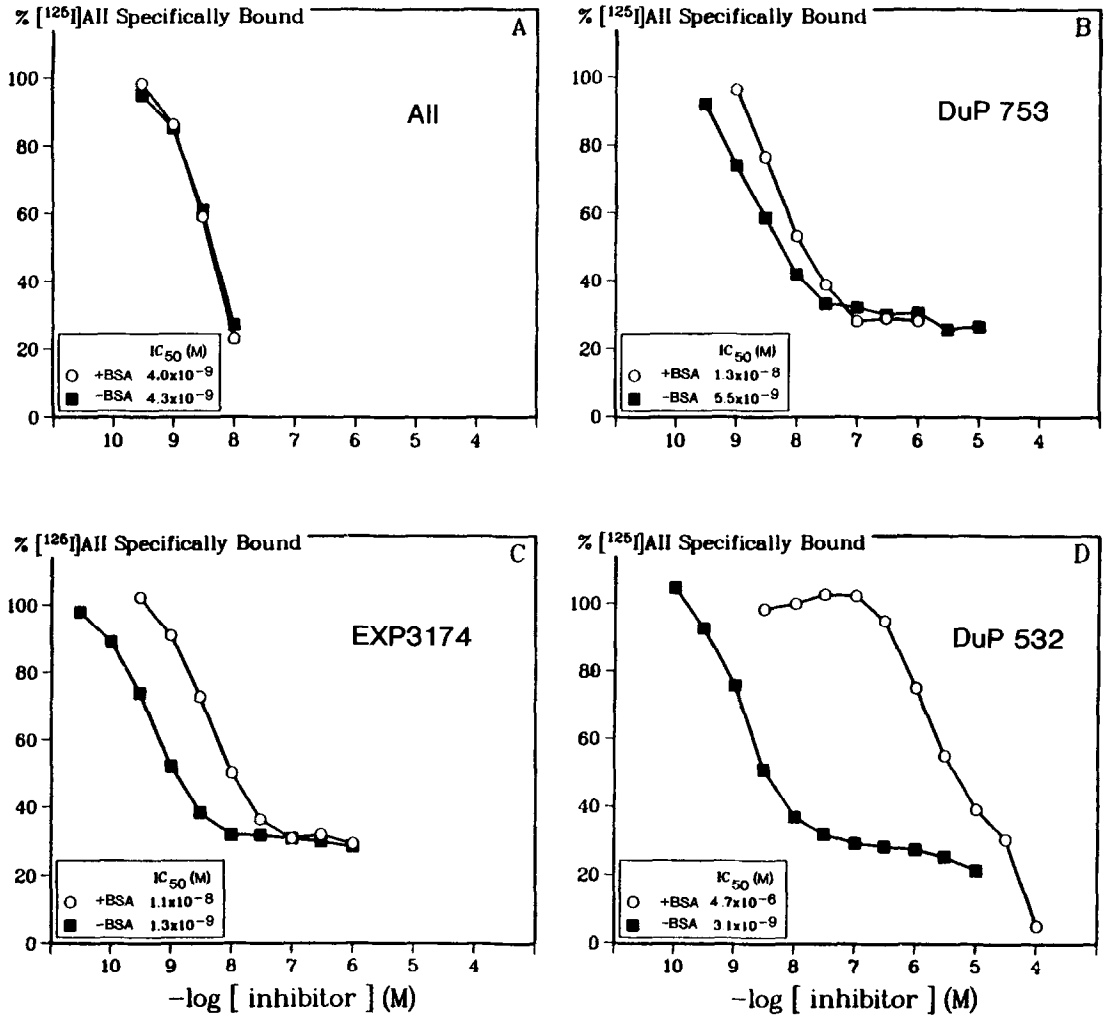


Fig. 2. Effect of the presence or absence of BSA (0.25%) on the inhibition of [^{125}I]AII (0.05nM) binding by AII (A), DuP 753 (B), EXP3174 (C) and DuP 532 (D) in rat adrenal cortical microsomes.

concentration-inhibition curve of DuP 532 was shifted to the left yielding an IC_{50} value of 6 x 10^{-9}M which amounts to an increase of 200-fold of the inhibitory potency (Fig. 4).

Direct binding to BSA. When BSA was absent in the buffer, [^{14}C]DuP 532 (2 x 10^{-9} to 2 x 10^{-6}M) , [^{3}H]DuP 753 (2 x 10^{-9} to 2 x 10^{-6}M) or [^{125}I]AII (0.5 x 10^{-9}M) all passed through the filter (data not shown). With BSA in the buffer, 99% of [^{125}I]AII passed through the filter whereas over 98% of [^{14}C]DuP 532 or about 70% of [^{3}H]DuP 753 at varying concentrations was retained on the filter (Fig. 5).

Specificity of the Diacidic analogs of nonpeptide AII receptor antagonists. EXP3174 and DuP 532 inhibited the [^{125}I]AII binding in the absence of BSA with IC_{50} values of 1.2 x 10^{-9}M and 3.0 x 10^{-9}M,

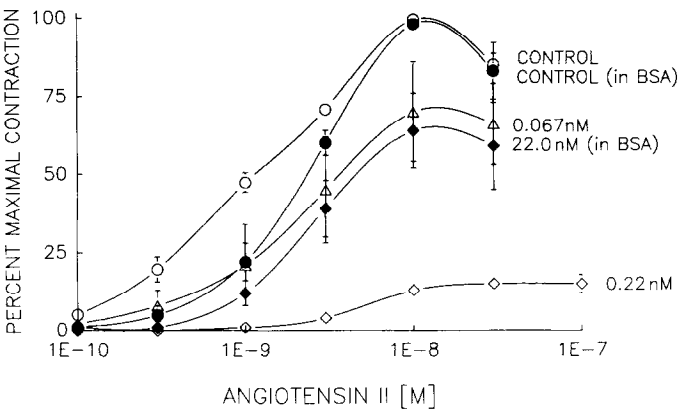
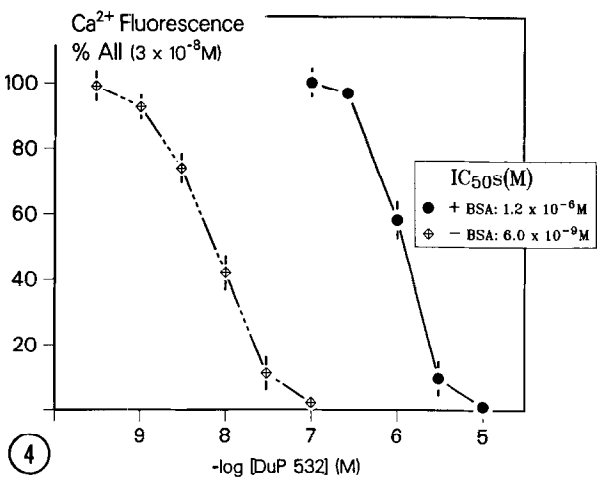


Fig. 3. Effect of BSA upon DuP 532 antagonism of contractile responses to AII in the isolated rat pulmonary artery. Each point represents the mean ± S.E. of at least 3 tissues. Solid and open symbols represent responses in the presence and absence of BSA (0.2%), respectively.

respectively. At concentrations up to 10^{-5}M, both showed no inhibition of [^{125}I]neurotensin or [^{3}H]prazosin binding in rat brain and [^{3}H]nitrendipine binding in rat cardiac membranes (data not shown).



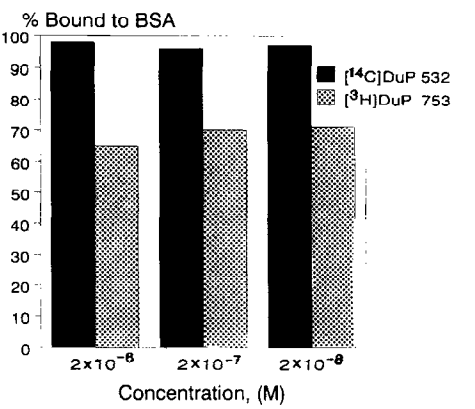


Fig. 4. Effect of BSA (0.5 mg/mL) on the inhibition of AII (3 x 10^{-8}M)-induced increases of Fura-2 fluorescence by DuP 532 in rat aortic smooth muscle cells. Cell suspensions were pretreated with varying concentrations of DuP 532 for 2 min before addition of AII, the stimulatory effect of which was taken as 100%. The data were presented as mean ± S.E. (n=4).

Fig. 5. Binding of [^{14}C]DuP 532 and [^{3}H]DuP 753 to BSA. After 60 min (same for 5 min) of incubation of radiolabels in the ligand-binding assay buffer containing 0.25% BSA, the amounts of radioactivity retained by the filters were presented as a % of the total added.

DISCUSSION

For the design of nonpeptide AII receptor antagonists, apparent receptor binding affinity provided the basis for structural optimization (2). Our initial experience indicated that there was a high degree of correlation between the inhibitory binding constants (K_is) and the in vitro functional inhibitory indices (pA_2s) even though the AII receptors in these preparations were derived from two different types of tissue and from different species (2,8). These early efforts led to the development of an orally active and highly potent AII antagonist, DuP 753 which targets specifically for the vascular-type of AII receptors, the AT_1 subtype (2,5,9). At present, the AII receptors that show high affinity for, or are blocked by, DuP 753 are classified as the AT_1 subtype and those that display preference for PD123177 and its analogs or for CGP 42112A are classified as the AT_2 subtype (10). Most of the well-known responses of AII are mediated through the AT_1 receptors. These include vasoconstriction, aldosterone secretion, non-vascular smooth muscle contraction, drinking, renal function and adrenal catecholamine release (7,11,12,13,14). In contrast, the functionality of AT_2 receptors remains to be delineated.

Another suggestion of further subdividing AT_1 receptors into two subclasses arose when a series of nonpeptide AII antagonists was found to deviate from the normal affinity-inhibitory potency relationship. Our initial observations indicated that these agents, DuP 532, EXP3174 and EXP3892, displayed far greater inhibitory potencies in rabbit aortic strips than would have been predicted by the apparent binding constants obtained from rat adrenal cortical microsomes. Structurally, they are close analogs of DuP 753 with the exception that they all share one distinguishable chemical characteristic as such having a carboxyl functionality at the 5-position of the imidazole ring. In other words, these are just the diacidic analogs of DuP 753. Is it plausible that they can differentiate the subclasses of the AT_1 receptors? According to our binding assessment using cultured smooth muscle cells from rat aorta (ATC: unpublished data) the results do not support such a speculation.

In order to resolve the discrepancy between the biochemical and the pharmacological parameters, the possibility of interfering factor(s) in the binding assay was considered. It is generally known that many drugs are bound to serum proteins, mostly to serum albumin, and as a result the free drug concentration is reduced at the locus of action (15). As noted, bovine serum albumin was a normal constituent in all our binding assays and was absent in the contractile assay. Therefore, the removal of BSA from the binding buffer was found to enhance the apparent binding affinity of DuP 532 by 1500-fold and that of EXP3174 by 20-fold. Interestingly, DuP 753 was much less affected, whereas the peptide ligand, AII, was unaffected by the absence of BSA. Reassessment of the binding for all diacidic analogs in the absence of BSA thus revealed a high degree of correlation between the apparent affinity constants and the indices of functional antagonism. Therefore, DuP 532 and its related analogs possess high affinity in the nanomolar range. These results also

suggest that the interference by BSA is rather selective for the diacidic nonpeptide analogs and its action is not at the level of the receptors.

Radiolabeled ligands confirmed the strong effect of protein binding on apparent AII receptor affinity. [^{14}C]DuP 532 binds rapidly to bovine serum albumin and the binding appears to be unsaturable. Over 98% of DuP 532 was bound at all concentrations from 10^{-9}M to 10^{-6}M. On the other hand, only 70% of [^{3}H]DuP 753 was bound to BSA at the same concentration range, whereas [^{125}I]AII showed no affinity for BSA.

The interference by BSA with the AII-induced contractile responses in rat pulmonary arteries and with AII-elicited Ca^{2+} mobilization in cultured smooth muscle cells from rat aorta could also be demonstrated in vitro. DuP 532 was much less potent when BSA is present whereas the AII effects were unchanged. Under these experimental conditions, the degree of enhancement of the response to AII was not as great as that obtained from binding. Nevertheless, the inhibitory constant obtained in the absence of BSA is quite comparable to that from the binding assay. The lesser effect of BSA on a functional assay may be due to the lower concentration of BSA used and/or the rapid pharmacokinetics of drug-receptor interaction.

DuP 532 inhibited the AII-induced responses by a noncompetitive mode of action, characterized by a shift of the response curve to the right with a depression of maximal response as previously described for EXP3174 and EXP3892 (11,16). Numerous speculative models (16,17) have been proposed in an attempt to explain the noncompetitive nature of this class of antagonists and of a number of hormonal receptor antagonists but there is no biochemical or biophysical evidence supporting their validity. Obviously, more work is needed to delineate such a mechanism. In preliminary biochemical studies, we examined the dissociation of [^{3}H]EXP3174 from AT_1 receptors in rat adrenal cortical microsomes. In comparison, EXP3174 dissociates from the AT_1 receptor at a rate 20 times slower than DuP 753, suggesting a possible pseudoirreversible mode of action. However, the situation in an intact tissue is much more complex than the membrane preparation. Therefore, other factors such as the distribution of the antagonist on and across the cell membrane, location and type of the AT receptor and production of endogenous mediators should be carefully examined.

In spite of the high binding towards BSA, DuP 532 and its analogs are in fact specific for the AT_1 receptor which is their primary pharmacological target of action. These agents do not interact biochemically and pharmacologically with other hormonal receptors such as neurotensin, alpha adrenoceptors, bradykinin, Ca^{2+} channels, and vasopressin. Therefore, the nonspecific binding to proteins per se may be regarded as storage depots or carriers which will ultimately influence the pharmacodynamics of drug action, especially in an in vivo situation. The in vivo pharmacology of DuP 532 will be discussed elsewhere. Suffice to say at this time that DuP 532 is an orally active and highly potent antihypertensive agent which displays a long duration of action in renal artery-ligated hypertensive rats (18).

In view of these biochemical and pharmacological characteristics, DuP 532 and related analogs may represent the second generation of

nonpeptide AII receptor antagonists which will shed further light on the integrity of the AT_1 receptors.

ACKNOWLEDGMENTS : The authors wish to thank Tam T. Nguyen and William A. Roscoe for their excellent technical performance.

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