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The In Vitro Binding Properties of Non-Peptide AT₁ Receptor Antagonists

P. M. L. Vanderheyden, I. Verheijen, F. L. P. Fierens, G. Vauquelin

A major breakthrough in the development of AT_1 receptor antagonists as promising antihypertensive drugs, was the synthesis of potent and selective non-peptide antagonists for this receptor. In the present manuscript an overview of the *in vitro* binding properties of these antagonists is discussed. In particular, CHO cells expressing human AT_1 receptors offer a well-defined and efficient experimental system, in which antagonist binding and inhibition of angiotensin II induced responses could be measured. From these studies it appeared that all investigated antagonists were competitive with respect to angiotensin II and bind to a common or overlapping binding site on the receptor. Moreover this model allowed us to describe the mechanism by which certain antagonists depress the maximal angiotensin II responsiveness in vascular contraction studies. Insurmountable inhibition was found to be related to the dissociation rate of the antagonist- AT_1 receptor complex. The almost complete (candesartan), partially insurmountable inhibition (irbesartan, EXP3174, valsartan) or surmountable inhibition (losartan), was explained by the ability of the antagonist-receptor complex to adopt a fast and slow reversible state. The equilibrium between both states depends on the structure of the antagonists (candesartan and EXP3174) as measurable in washout experiments, may contribute to a long-lasting blood pressure lowering effect *in vivo. J Clin Basic Cardiol 2002; 5: 75–82.*

Key words: angiotensin II, AT1 receptor, CHO cells, insurmountable, surmountable

t has been established that the renin-angiotensin system plays a central role in the regulation and the management of hypertension [1, 2]. Angiotensin II, the major player in this system, elicits a wide array of biological actions, including vascular smooth muscle contraction and growth of smooth muscle cells and cardiac myocytes [3]. These effects are triggered by activation of angiotensin type 1 receptors [4]. As these receptors play a major role in the regulation of cardiovascular homeostasis, the development of non-peptide AT₁ receptor antagonists represents a very important contribution in the effective treatment of hypertension and congestive heart failure [5-8]. Among the numerous antagonists, a few non-peptide AT1 antagonists (ARB) are approved by the Food and Drug Administration and available for the treatment of hypertension [9]. These include losartan, valsartan, irbesartan, eprosartan and candesartan cilexetil [9]. On the basis of their structure, all, except eprosartan, have a common

Table 1. Pharmacological properties of AT1 receptor antagonists

biphenyl-tetrazole ring structure, but they differ in their side chains. In the present review, we will discuss how the receptor binding properties of the biphenyl-tetrazole containing antagonists, as extensively studied in *in vitro* models, may contribute to their pharmacodynamic as well as *in vivo* binding properties.

The pharmacological characteristics of these ARBs are summarized in Table 1. The most striking difference in *in vitro* studies emerges from concentration-response studies in which the angiotensin II induced contraction of the rabbit aorta was measured. Based on differences in their ability to depress the maximal response to angiotensin II the ARBs are commonly divided into two categories: The first category comprises antagonists that, when preincubated with the tissue, cause a rightward and parallel shift of the angiotensin II concentration-response curve. These antagonists include losartan and eprosartan and are denoted as competitive or

AT ₁ antagonist	Candesartan cilexetil	Eprosartan	Irbesartan	Losartan	Valsartan	Telmisartan
Active metabolite	Candesartan	No	No	EXP3174	No	No
AT ₁ receptor antagonism	Insurmountable	Insurmountable	Insurmountable	Surmountable (losartan) Insurmountable (EXP)	Insurmountable	Insurmountable
Maximal depression Emax	95–100 %	Not detectable	30 %	Not detectable 60–70 % (EXP)	50–55 %	50 %
% protein binding	99.5 %	98 %	90 %	98.7 % (losartan) 99.8 % (EXP)	95 %	> 98 %
Doses available (mg)	4, 8, 16, 32	200, 300, 400	75, 150, 300	25, 50	80, 160	48, 80
Starting dose (mg)	16	600	75–150	25–50	80	40
Plasma half-life	9–12 h	5–7 h	11–15 h	2 h (losartan) 6–9 h (EXP)	6 h	24 h

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surmountable antagonists [10–12]. The second category includes antagonists that, when preincubated with the tissue, depress the maximal response to angiotensin II. They are classified as insurmountable, non-surmountable or non-competitive antagonists. The degree to which the maximal response is reduced is variable and ranges from partial for irbesartan, valsartan, telmisartan and EXP3174 (the more active metabolite of losartan) to almost complete for candesartan (the active metabolite of candesartan cilexetil) [13–17]. Several theories have been put forward to explain the differences of the inhibitory pattern of the ARBs at the molecular level. Such theories may have important consequences for the interpretation of *in vitro* binding data as well as the correlation between *in vivo* receptor occupancy and the long-lasting reduction of blood pressure by some of the ARBs.

In the last three years we have set up an *in vitro* pharmacological model using CHO cells that are stably transfected with the gene for the human AT_1 receptor (CHO-h AT_1 cells). Similar to primary cultured vascular smooth muscle cells, angiotensin II could be shown to activate the phophoinositide signalling system in CHO-h AT_1 cells. The parallel measurement of angiotensin II stimulated inositol phosphate formation and the [³H]-antagonist binding to intact CHO-h AT_1 cells allowed us to elucidate and unravel the binding properties of the biphenyltetrazole containing ARBs candesartan, irbesartan, valsartan, and losartan and its more active metabolite EXP3174. The results of this work enabled us to tackle the following questions:

- (i) Are all ARBs competitive AT₁ receptor antagonists?
- (ii) Is there a relationship between insurmountable antagonism and slow dissociation from the receptor?
- (iii) Is there a link between insurmountable antagonism, the structure of the antagonists and the molecular structure of the AT₁ receptor?
- (iv) What are the repercussions of the in vitro binding data?

Are All ARBs Competitive AT₁ Receptor Antagonists?

Until recently, the 'in vitro' pharmacological properties of ARBs were usually studied by comparing their inhibition of angiotensin II induced contraction of rabbit aorta with radioligand binding on rat adrenal, lung, liver or kidney membranes [15, 18-24]. Theoretically, this approach encounters two major problems. The binding properties of AT₁ receptors of two different species are compared and are extrapolated to be similar with the human receptors. Moreover, the binding properties of ARBs may be essentially different in tissues versus cell membranes. To circumvent these potential problems an in vitro model using CHO cells that are permanently transfected with the coding region for the human AT1 receptor (CHOhAT1 cells) was developed [25]. As in primary cultures of vascular smooth muscle cells, activation of the recombinant hAT₁ receptor leads to the accumulation of inositol phosphate (IP) and a transient elevation of the intracellular free calcium concentration. In a first series of experiments, antagonist inhibition of angiotensin II responses was studied in experimental conditions similar to those in aorta contraction experiments. For this purpose CHO-hAT₁ cells were preincubated with the investigated antagonist (for 30 min) and then challenged with increasing concentrations of angiotensin II (5 min). Under these conditions, the investigated AT₁ antagonists cause along with a rightward shift of the concentration-response curve a depression of the maximal angiotensin II response (Emax) to a varying degree; 90-95 % for candesartan, 60-70 % for EXP3174, 55 % for valsartan, 30 % for irbesartan and not detectable for the typical surmountable antagonist

losartan (Fig. 1). Comparable fully and partially insurmountable antagonism, with the same degree of depression of the Emax for each ARB, is reported for aorta contraction studies [10, 11, 15–17]. Whereas this nicely illustrates the ability to distinguish surmountable and insurmountable antagonists on CHO-hAT₁ cells, the question remains whether such inhibitory pattern is the result of a (non-)competitive type of interaction of certain ARBs with the AT₁ receptor. This terminology refers to the (in)ability of antagonists to affect the binding of an agonist to the receptor. Indeed, non-competitive antagonists may cause a depression of the maximal response. However, the same inhibitory pattern may also be observed with irreversible or slow-dissociating competitive antagonists. When tissues or cells are preincubated with such antagonists, the generally short presence of the agonists would be insufficient to overcome the antagonist inhibition. Such a situation is denoted as a hemi-equilibrium and results in a depression of the maximal response. On the other hand, the more generally known behaviour of competitive antagonist, ie causing a parallel rightward shift of the agonist concentration response curve, occurs for fast dissociating reversible antagonists only. To resolve this issue with respect to the insurmountable and surmountable AT₁ receptor antagonists, both the antagonist and angiotensin II have to be applied simultaneously to the receptor. This implies that pre-equilibration of the tissue with the antagonist and cumulative dosing of angiotensin II, as carried out in contraction studies, should be avoided. When such so-called 'co-incubation' experiments are carried out, competitive antagonists will not affect the maximal response, whilst non-competitive antagonists would still decrease the maximal response. The same principle is valid in radioligand saturation binding experiments. When a competitive ligand is co-incubated with the radioligand it will not affect the maximal binding, whereas co-incubation (or pre-incubation) with a non-competitive ligand will result in a reduction of the maximal binding capacity. Accordingly concentration-response curves were generated from IP accumulation experiments in CHO-hAT1 cells in which the antagonists and angiotensin II are applied simultaneously (Fig. 2). From this figure it is clear that all antagonists have a similar influence on the angiotensin II concentra-



Figure 1. Concentration-response curves of angiotensin II induced IP production (5 min at 37 °C) after a pre-incubation for 30 min with candesartan (a), EXP3174 (b), irbesartan (c) or losartan (d). By friendly permission of Nature Publishing Group from [25].

tion effect curve, ie, they all cause a parallel rightward shift, without changing the maximal response. A similar pattern was observed by Criscione et al. when examining the effect of valsartan. Pre-incubation of aortic strips with valsartan resulted in a partial reduction of the maximal contraction elicited by angiotensin II. On the other hand, the concentration response curves of angiotensin II induced aldosterone release in bovine adrenal glomerulosa cells were rightward shifted when valsartan was applied simultaneously with angiotensin II [14]. From these observations it is obvious that the ability of certain antagonists to depress the maximal angiotensin II response is a consequence of the experimental condition (ie antagonist pre-incubation) and cannot be ascribed to a noncompetitive behaviour. Subsequently direct radioligand binding experiments with [3H]-candesartan on intact CHOhAT₁ cells were carried out in the absence or simultaneous presence of unlabelled EXP3174, irbesartan or losartan [26]. It appeared that all investigated AT₁ antagonists failed to decrease the maximal binding of [3H]-candesartan. In the same line as the functional experiments these finding provided convincing evidence that the different investigated AT₁ antagonists bind to the same or overlapping binding sites on the receptor. In summary, the experimental data strongly suggest that the different ARBs are competitive AT1 antagonists and that insurmountable inhibition of some of them can be ascribed to long-lasting or even irreversible binding to the AT₁ receptor.

Is There a Relationship Between Insurmountable Antagonism and Slow Dissociation From the Receptor?

To investigate and quantify the dissociation rate of the AT_1 antagonists, three experimental approaches were followed. In the first approach, CHO-hAT₁ cells are pretreated with an antagonist, washed and than further incubated for different time periods in the washout medium, after which the angiotensin II induced IP production is measured. None of the in-



Figure 2. Concentration-response curves of angiotensin II induced IP production (5 min at 37 °C) in the simultaneous presence of candesartan (a), EXP3174 (b), irbesartan (c) or losartan (d). Reprinted from Eur J Pharmacol, 372, Fierens FLP et al., Insurmountable angiotensin AT₁ receptor antagonists: the role of tight antagonist binding, 199–206, © 1999, by friendly permission of Elsevier Science, [36].

vestigated AT1 receptor ligands bind irreversibly. On the other hand, the rate to which the functional responses recover is variable among the different antagonists (Fig. 3a). Whereas the half-maximal recovery after candesartan preincubation was about 5 hours, it was faster after EXP3174 (44 min), valsartan (25 min), irbesartan (18 min) and almost instantaneous after losartan pre-treatment. Whilst these findings are compatible with a slow dissociation of insurmountable antagonists, it does not necessarily imply that the antagonists exert their long-lasting inhibition by binding to the receptor. In this context a slow interconversion between an inactive and active receptor conformation, much slower than the ligand binding, is proposed in a two-state model by Gero [27] and Robertson et al. [28] and in the related coupling model of de Chaffoy de Courcelles et al. [29]. To address this issue the rate of functional recovery from antagonist inhibition was compared with the time course of [3H]-candesartan, [3H]valsartan and [³H]-irbesartan dissociation, both initiated by washing of the cells [30-32]. As shown in Figure 4a and Table 2 it appeared that the rate by which these radioligands dissociated completely matched the recovery in the functional experiments. As a consequence of the parallel between the binding and the functional inhibition by the investigated ARBs, there is no experimental ground to assume that insurmountable action by these antagonists is related to other models than their slow dissociation from the receptor. Interestingly, inclusion of losartan in the washout medium caused a 4- to 5-fold increase of rate of the functional recovery after candesartan preincubation (Figs. 3b and 4b). A similar increased dissociation rate was seen in the [3H]-candesartan washout experiments not only by adding losartan, but also



Figure 3. Recovery of angiotensin II induced IP production after antagonist pre-incubation of CHO-hAT₁ cells and subsequent wash-out either in the absence (a) or presence of 1 μ mol/l losartan (b) for the indicated periods of time. Reprinted from Biochem Pharmacol, 59, Vanderheyden PML et al., Reversible and syntopic interaction between angiotensin receptor antagonists on Chinese Hamster Ovary cells expressing human angiotensin II type 1 receptors, 927–35, © 2000, by friendly permission of Elsevier Science, [26].

other AT₁ receptor ligands in the washout medium. These findings can be explained by the ability of candesartan to accumulate in the washout medium and to re-associate to the receptor due to a combination of its extremely high affinity ($K_D = 51 \text{ pM}$) and a relatively high receptor density in CHO-hAT₁ cells [30]. When a high concentration of unlabeled antagonist is included in the washout medium, this rapidly occupies AT1 receptors that become available after dissociation of [³H]-candesartan, resulting in an apparently increased dissociation rate of the radioligand. Quantitatively the inhibitory action of candesartan decayed with a half-life of about 2 instead of about 5 hours in washout experiments when losartan was included in the washout medium. A similar losartan mediated increase of the decay of EXP3174 inhibition was seen (the half-life is 30 min in the presence of losartan as compared to 45 min). On the other hand, no perceptible re-binding occurs for irbesartan. The phenomenon of re-binding is not unique for AT1 receptor antagonists, as it has also been observed in several studies on the interaction of high affinity ligands to cell surface receptors [33, 34]. Whereas it has not proven to occur in vivo, it is to be expected that the re-binding of certain ARBs may contribute to the duration of their antihypertensive effects.



Figure 4. Comparison of functional recovery from candesartan preincubation (open symbols) and dissociation of [³H]-candesartan binding (closed symbols) to CHO-hAT₁ cells. The antagonist preincubation is followed by washout either in the absence (a) or presence of 1 µmol/l losartan (b) for the indicated periods of time. Reprinted by friendly permission of Elsevier Science from Biochem Pharmacol, 59, Vanderheyden PML et al., Reversible and syntopic interaction between angiotensin receptor antagonists on Chinese Hamster Ovary cells expressing human angiotensin II type 1 receptors, 927–35, © 2000, [26], and from Eur J Pharmacol, 372, Fierens FLP et al., Insurmountable angiotensin AT₁ receptor antagonists: the role of tight antagonist binding, 199–206, © 1999, [36].

The third method to determine the dissociation rate of the ARBs in vitro is by measuring the slowing of the association rate of [3H]-candesartan after pre-treatment of the CHOhAT₁ cells of a certain antagonist. This method has been described previously by Hara et al. and is not 'biased' by the phenomenon of re-binding [35]. The corresponding rate constants reflect the 'true' dissociation of the antagonist from the receptor and should be compared with functional recovery and [3H]-antagonist dissociation experiments in the presence of losartan. It appeared that the dissociation rate constants of the antagonists were independent of the experimental conditions and are summarized in Table 2 [26]. It is obvious that the relatively long half-life of the candesartan- and EXP3174-AT₁ receptor complex is largely adequate to explain the depression of the maximal angiotensin II responses in CHO-hAT1 cells as well as aorta contraction studies. On the other hand the dissociation of irbesartan and valsartan is faster, but still sufficient to explain their partial insurmountable inhibition.



Figure 5. Antagonist concentration inhibition curves (a) CHO-hAT₁ cells were pre-incubated for 30 min with increasing antagonist concentrations, followed by a 5 min challenge with 10 μ mol/l angiotensin II. Curves are calculated from the two-state two-step mechanism using the parameters given in Table 3 (b) Pre-incubation of the cells with irbesartan followed by a 5 min incubation with the indicated concentration of angiotensin II. Reprinted from Eur J Pharmacol, 372, Fierens FLP et al., Insurmountable angiotensin AT₁ receptor antagonists: the role of tight antagonist binding, 199–206, © 1999, by friendly permission of Elsevier Science, [36].

Table 2. Dissociation rate of non-peptide AT₁ receptor antagonists. The half-lives in min., and the corresponding kinetic constants (given in between parentheses in min⁻¹) were derived from (a) slowing of the [³H]-candesartan association rate after pre-treatment of the CHO-hAT₁ cells with 1.5 nmol/l candesartan, 5 nmol/l EXP3174, 100 nmol/l irbesartan and 10 μ mol/l losartan, washing of the cells and further incubation with 1.5 nM [³H]-candesartan [26] and (b) recovery from insurmountable inhibition of angiotensin II (0.1 μ mol/l) induced IP accumulation by AT₁ antagonists (same concentrations as slowed association) and washout of the cells with DMEM either without or containing 1 μ mol losartan [26] and (c) by direct binding of 1.5 nmol/l [³H]-candesartan [30], 3 nmol/l [³H]-irbesartan [31] or 1.5 nmol/l [³H]-valsartan [32]. n.m. = not measurable; n.d. = not determined

	[³ H]-candesartan association rate	Functional recovery		Direct [³ H]-antagonist binding		
		Washout alone	Washout+losartan	Washout alone	Washout+losartan	Isotopic dilution
	t _{1/2} (k ₋₁)	t _{1/2} (k ₋₁)	t _{1/2} (k ₋₁)	t _{1/2} (k ₋₁)	t _{1/2} (k ₋₁)	t _{1/2} (k ₋₁)
Candesartan	152 ± 58 (0.004)	315 ± 43 (0.002)	185 ± 46 (0.003)	693 ± 69 (0.001)	151 ± 4 (0.005)	112 ± 2 (0.006)
EXP3174	31 ± 6 (0.022)	44 ± 7 (0.016)	$33 \pm 11 \; (0.021)$			
Irbesartan	17 ± 4 (0.040)	$18 \pm 6 \; (0.039)$	12 ± 6 (0.058)	6.0 ± 1.2 (0.128)	$6.4\pm0.5(0.101)$	$6.7\pm0.3\;(0.104)$
Valsartan	n.d.	n.d.	n.d.	$25 \pm 1 \ (0.028)$	14 ± 1 (0.049)	17 ± 2 (0.041)
Losartan	5.2 ± 1.1 (0.13)	n.m.	n.m.	n.d.	n.d.	n.d.

The observation remains an unresolved issue that some AT₁ antagonists such as irbesartan, EXP3174 and valsartan, only partially depress the maximal angiotensin II response. The degree of their insurmountable inhibition is similar in aorta contraction studies and on CHO-hAT1 cells. It appears that the level of such insurmountable inhibition is an intrinsic property of the antagonist. To better quantify this, CHOhAT₁ cells were pre-incubated with increasing antagonist concentrations, followed by a challenge of angiotensin II (0.1, 1 or 10 μ mol/l). The resulting antagonist concentration-inhibition curves were clearly biphasic and a typical example of such curves is shown in Figure 5a when the 10 µmol/l angiotensin II induced IP production was measured. Whereas the most potent component (ie the insurmountable part) was independent of the angiotensin II concentration, the IC₅₀ of the less potent component was agonist independent (ie the surmountable part) (Fig. 5b). The proportion of insurmountable versus surmountable inhibition (in CHO-hAT₁ cells) was about 94 % for candesartan, 70 % for EXP3174, 55 % for valsartan, 30 % for irbesartan and not detectable for losartan [31, 36]. The degree of insurmountable inhibition was not affected by varying the pre-incubation time with the antagonist. When assuming a single bimolecular interaction between the antagonist and the receptor, such a partially insurmountable inhibition cannot be solely explained by the dissociation rate of the antagonist. In a recent study it was proposed that in addition to the antagonist dissociation rate, the difference between the receptor desensitization and resensitization rate would determine the maximal depression of the angiotensin II response [37]. Functional models in which these phenomena occur are those in which the agonist typically elicits a transient response. According to this proposal the receptor desensitization can be the result of rapid agonist induced receptor phosphorylation, response fading or depletion of the free intracellular calcium by the sarcoplasmatic reticulum that may take place in transient calcium responses. A rapid and transient response can indeed be seen in CHO-



Figure 6. Two-state two-step model for describing the interaction of non-peptide antagonists with AT_1 receptors

hAT₁ cells, when measuring the angiotensin II induced increase of intracellular calcium concentration. On the other hand, angiotensin II induced IP accumulation increases linearly with time, by an up to 10 min incubation suggesting there is no noticeable desensitization and/or fading of the IP accumulation response during the time-scale of the functional experiments [25]. Clearly the same partially insurmountable antagonism has been seen with valsartan for both angiotensin II induced IP accumulation as well as the transient rise in intracellular calcium concentration in CHOhAT₁ cells [32]. In the same line the degree of insurmountable inhibition by this antagonist has not been affected by measuring angiotensin II induced IP accumulation in the presence of sucrose, which prevents internalisation and as a consequence receptor resensitization (unpublished data, 2001). As the proposed dynamic model assumes that fully and partially insurmountable inhibition can only be seen in models with transient responses it is clearly not applicable for the results in CHO-hAT₁ cells.

An alternative explanation for the partially insurmountable antagonism by the investigated AT_1 antagonists is proposed by a two-state two-step model [36]. In this model, the initial binding of all antagonists is very fast and reversible (ANT-R). This binding becomes insurmountable if the antagonist-receptor complex is further converted into a tight binding/slow reversible state (ANT-R*). Equilibrium between both states is rapidly achieved and is dependent on the structure of the antagonist. In the case of antagonists such as losartan, the tight binding state is not formed, so that the binding remains surmountable (Fig. 6). On the hand, insurmountable antagonists can induce this conversion to a vary-

Table 3. Parameters of the antagonist binding to the AT₁ receptor for the computer-assisted simulations. These match the curves shown in Figure 5. These kinetic parameters or ratios are calculated according to a single-state model for the surmountable antagonist losartan and according to a two-state, two-step model for the insurmountable antagonists irbesartan, EXP3174 and candesartan

Kinetic constants / ratios	k ₋₁ / k ₁	k ₂	k.2
(Unit)	(nmol/l)	(M ⁻¹ .min ⁻¹)	(min ⁻¹)
Losartan	7.5		
Irbesartan	1.5	0.077	0.104
EXP3174	2.2	0.084	0.022
Candesartan	2.5	0.121	0.0061

ing degree. Hence the proportion of the tight binding or insurmountable state reflects the intrinsic effectiveness of a certain antagonist to induce the ANT-R to ANT-R* conversion. A similar terminology has been put forward to explain the existence of partial and full agonists [38]. In this case, an inactive and active state of the receptor has been proposed. With regard to the AT1 antagonists both ANT-R and ANT-R* appear to reflect both "inactive" conformations of the receptors, since none of the antagonists affect the basal IP accumulation in CHO-hAT₁ cells. The proposed two-state model can be described by the kinetic constants k1 and k-1 for the formation of the loose binding/fast reversible antagonist-receptor complex as well as the kinetic constants k₂ and k₋₂ related to the conversion of this complex to the tight binding/slow reversible state. Using a broad range of kinetic constants and taking into account the experimental dissociation rate of each antagonist, computer generated data were compared with experimental data points (ie antagonist concentration inhibition curves as shown in Fig. 5) [39]. The following conclusions could be attained when these computer-generated data match the data points from antagonist concentration inhibition experiments (Tab. 3):

- The k₂ values and the ratio k₋₁/k₁ are very similar for candesartan, irbesartan, EXP3174 as well as losartan.
- The k₋₂ values are in the same range as the experimental dissociation rates for candesartan, irbesartan and EXP3174.

Taken together these computer simulations indicate that the existence of (at least) two antagonist-receptor complexes is sufficient to explain nearly fully insurmountable (candesartan), partially insurmountable (irbesartan and EXP3174) and surmountable (losartan) inhibition of angiotensin II responses. The extent to which the maximal angiotensin II response is depressed is the outcome of differences in the stabilization of the tight antagonist-receptor complex reflected in the different k₋₂ values for the different antagonists.

Whereas the two-state model shown above accurately described the experimental antagonist inhibitory data, an outstanding issue that needs to be resolved is the molecular and/ or structural nature of the fast reversible and the tight binding/slow reversible antagonist-receptor states. Among the various hypothetical possibilities that can be formulated, the role of antagonist induced receptor internalisation may be expected to be involved. Indeed AT₁ receptor internalisation via coated pit formation has been shown to occur after angiotensin II binding [40]. After internalisation the receptors can be either degraded by lysozymal proteases or they are rapidly recycled onto the cellular membrane. If the latter process were to take place for non-peptide antagonists, one could hypothesize that the internalised receptor would not be accessible for angiotensin II and reflect the tight binding/slow reversible antagonist - AT1 receptor complex. This hypothesis was previously put forward to explain the insurmountable inhibition of synthetic peptide AT₁ receptor antagonists [41]. Furthermore, non-peptide antagonist induced internalization has been demonstrated for other G-protein coupled receptors such as CCK receptors expressed in CHO cells [42]. To investigate this proposition, the insurmountable inhibition and the direct binding of [3H]-candesartan to CHO cells expressing a C-terminal truncated rat AT_{1A} receptor (CHO-TL₃₁₄-rAT_{1A} cells) was studied. This mutated receptor has previously shown to have an impaired capacity of angiotensin II induced internalization. Contradictory to the suggested role of receptor internalisation in antagonist binding, the inhibition of angiotensin II induced IP accumulation by preincubation of these cells with candesartan remained almost completely insurmountable [43]. This inhibitory pattern as well as the dissociation rate of $[{}^{3}H]$ -candesartan was identical for rat AT_{1A} as well as human AT1 receptors. In the same line, visualization of GFP tagged AT_{1A} receptors in CHO cells did not internalised after pre-incubation of the cells with candesartan [44]. It is therefore unlikely that the tight AT₁ antagonist receptor complexes reflect internalised binding sites. An alternative possibility remains that the observed tight antagonist binding to AT₁ receptor can be attributed to the interaction of the receptors with other proteins, or the ability to form dimers. In this context G-protein dependent high affinity antagonist binding has been shown to occur for μ opioid receptor [45]. Because of the entirely speculative nature of these possibilities, it is obvious that further research is needed to investigate the structural and/or physical basis for the different antagonist-receptor states for ARBs.

Is There a Link Between Insurmountable Antagonism, the Structure of the Antagonists and the Molecular Structure of the AT_1 Receptor?

It is obvious that the strength with which the investigated ARBs bind to the AT₁ receptor is associated to their chemical structure. Whereas losartan only possesses one acidic tetrazole moiety, many insurmountable AT₁ receptor antagonists such as candesartan and EXP3174 have an additional acidic carboxylate group. It can be reasoned that a positively charged amino acid at the receptor may serve as a counter ion for such a critical functional group. Such a strong interaction may stabilize slow reversible antagonist-receptor complexes and produce insurmountable inhibition. Site-directed mutagenesis studies involving the substitution of certain basic amino acid residues of the AT₁ receptor offer a way to evaluate this hypothesis.

The observation in previous mutation work that Lys199 was found to be involved in the interaction with the end-standing carboxylate of angiotensin II and of the peptide AT1 antagonist sar¹ile⁸-angiotensin II made it a good candidate for the nonpeptide antagonist binding as well [46]. Indeed the substitution of Lys199 by a non-charged Gln indeed decreased the binding affinity of non-peptide antagonists [47]. Interestingly this decrease in affinity was proportional to the degree of insurmountable inhibition. It led us to conclude that Lys199 in the AT₁ receptor appears to be involved in the formation and/or stability of the tight binding/slow reversible antagonistreceptor complex. Referring to the antagonist's structure this implies that the presence of and correct positioning of a second acidic (and possibly a carboxylate) group is pivotal for the tight binding of an antagonist to the AT₁ receptor. In line with this, many other non-peptide AT₁ receptor antagonists that produce an insurmountable inhibition are also di-acidic molecules such as valsartan, GR117289, BMS-180560, CI-996, LR-B/ 057, KRH-594 and the 6-carboxylate derivative of 5Hpyrazolo[1,5-b][1,2,4]triazole [13, 14, 21-24].

As outlined above, the two-state, two-step model is applicable for an antagonist with a common biphenyltetrazole moiety. On the other hand it is not clear whether this model is also applicable to non-peptide AT_1 antagonist with a dissimilar structure. In this context the polysubstituted 4aminoimidazole derivatives LY301875 and LY303336 are synthesized to possess three ionisable groups at physiological pH and three chiral centers [48, 49]. These compounds were reported to produce an antihypertensive activity after oral application and, when pre-incubated with rabbit aorta, they produce insurmountable inhibition of angiotensin II induced contraction. Based on their structure it was suggested that these antagonists might bind to a distinct subsite of the AT_1 receptor that is different to that for losartan. As in the contraction studies pre-incubation of CHO-hAT₁ cells with LY301875 and LY303336 caused an insurmountable inhibition of angiotensin II induced IP accumulation. As for the biphenyltetrazole antagonists this insurmountable effect was related to slow dissociation from the AT₁ receptor [50]. Moreover, the partial insurmountable inhibition is compatible with the ability of LY301875 and LY303336 to form the insurmountable/long lasting as well as an insurmountable/ fast reversible complex with the AT₁ receptor [50].

What are the Repercussions of the In Vitro Binding Data?

The relative impact of the *in vitro* binding properties of AT₁ receptor antagonists is important in the understanding of the duration of the antihypertensive activity of certain of these drugs. In the case of candesartan, a link between the slow receptor dissociation combined with its re-binding to the receptor and its long-lasting effect on more classical contraction models as well as its blood pressure lowering effect seems to be likely but remains to be established. In this context washout experiments with candesartan-treated rabbit aortic strips and rat portal vein sections, revealed that the decline of the contractile response is still severe after several hours [51]. In such tissue experiments the phenomenon of 'rebinding' provides an attractive explanation for this very long lasting effect of candesartan. Moreover, it could provide a rationale for the observation that, following treatment of aortic strips with insurmountable antagonists such as GR117289 and candesartan, the recovery of the contractile function is accelerated when surmountable antagonists such as losartan are present in the washout medium [13, 52]. A prolonged antihypertensive action of candesartan cilexetil has also been established in clinical studies. In this respect, it was recently shown by Lacourcière et al. [53] in a double-blind, forced titration study in ambulatory hypertensive patients that candesartan cilexetil is superior to losartan in reducing systolic arterial blood pressure and in controlling both systolic and diastolic arterial blood pressure on the day of a missed dose. In the same line, following the oral administration of candesartan cilexetil to healthy volunteers, an anticlockwise hysteresis loop could be observed when comparing the plasma concentration of candesartan with the inhibition of the pressor response to angiotensin II. This loop reflects a slow onset of the inhibitory effect of candesartan while its plasma concentration is increasing, and a sustained effect when its concentration is falling [54]. The most probable explanation of this disproportion between the anti-hypertensive action of candesartan and its plasma concentration may reside in the combination of the slow dissociation rate of candesartan along with its rebinding to the AT1 receptor as has been described in the in vitro binding studies in CHOhAT₁ cells. With respect to the *in vitro* results of EXP3174, the active metabolite of losartan, and of irbesartan and valsartan, their dissociation rate from the receptor is adequate to produce partial insurmountable inhibition on CHO-hAT₁ cells as well as in contraction studies. On the other hand the in vitro half-life is relatively short when compared with the documented long lasting anti-hypertensive effect of these antagonists [15, 55-57].

Further research on the study of the AT_1 receptor binding of non-peptide antagonists will therefore have to focus on potential factors that contribute to long-lasting inhibition of the AT_1 receptor such as local accumulation in certain tissues or in the extracellular matrix. In addition, the proposed twostate receptor model developed on the basis of data from CHO-hAT₁ cells also awaits validation in cells that endogenously express AT_1 receptors such as vascular smooth muscle cells or other target cells of the renin-angiotensin system. Ultimately the *in vivo* receptor occupancy should be correlated with the blood pressure lowering effects of AT_1 receptor antagonists.

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