Angiotensin II dose–effect curves and Schild regression plots for characterization of different angiotensin II AT_1 receptor antagonists in clinical pharmacology

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The 'Schild regression' method is based on the principle of assessing the rightward shift of agonist dose–effect curves in the presence of different doses/concentrations of the respective receptor antagonist and presenting their relationship in a double log plot (i.e. the 'Schild plot'). The original method was developed to quantitatively characterize antagonistic drugs in experimental pharmacology. The method was adopted for evaluation of various AT_1 antagonists in humans utilizing (human) angiotensin II as the agonist. Angiotensin II (Ang II) in continuous intravenous dose-incremental administration resulted in a clearly dose-dependent increase in blood pressure. All AT_1 antagonists tested after oral administration yielded concentration-dependent rightward shifts of those Ang II dose–effect curves that were quantified as dose ratio (DR). DR minus 1 (DR−1) enabled the assessment of antagonist time kinetics in humans and a quantitatively precise determination of the half-life of antagonism *in vivo*. Schild plots allowed for assessment of apparent K_i doses indicative of a twofold rightward shift of the Ang II effect, thus providing the means for a rational comparison of the pharmacological potency of many of these compounds, where the K_i doses obtained at 24 h after administration were in the range of 'therapeutic' doses. Schild plots of a variety of substances showed linear relations independent of whether the blockade was deemed surmountable or not. It is therefore assumed that this property does not play a role at clinical doses/concentrations. Slopes slightly below 1 in the Schild plots of all tested antagonists point to a second 'counterregulatory' vasodilatory mechanism of action of Ang II which becomes apparent with AT_1 blockade in conditions of high doses/concentrations of Ang II. Concentration *vs.* effect relationships indicate that if assessed at the same degree of direct vascular antagonism, other effects, such as increase in plasma renin activity, may be present to a varying degree with different antagonists. Thus for irbesartan, the potency to stimulate renin release was found to be at least twice that of candesartan. These observations should stimulate further research into the relevance of these dynamic differences between the various compounds. Thus, methodologies relying on fundamental principles of experimental pharmacology can provide the clinical pharmacologist with powerful tools to measure accurately degree of antagonism and time kinetics and to investigate the nature of receptor antagonism in humans.

Keywords: angiotensin II, AT_1 antagonist, dose–effect curves, pharmacodynamics, renin angiotensin aldosterone system, Schild regression plot

Antagonists of the angiotensin II (Ang II) AT_1 subtype receptor have been shown to be highly effective in treating hypertension, preventing development of diabetic nephropathy, and reducing cardiovascular morbidity and mortality [1–5]. Moreover, they are promising in the treatment of heart failure and atherosclerosis.

Today, a wide range of chemically different Ang II receptor antagonists are available. As their pharmacological properties are not identical, it is useful to develop their differential application. However, since all compounds act at the AT_1 receptor site, it proves difficult,

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except for pharmacokinetics and potency, to distinguish the agents from one another [6]. Furthermore, it is still a matter of conjecture whether or not differences in effects of these substances exist and what their significance could be [6, 7]. In terms of their pharmacokinetic properties in humans, AT_1 antagonists have been shown to differ in parameters such as half-life ($t_{1/2}$) and volume of distribution (Vd) [8]. It is therefore assumed that pharmacokinetic properties resulting in differences in distribution to tissues (compartmentalization) are the main source of secondary pharmacodynamic effects and variations. Variations in Vd indicate different concentrations of the antagonists in various body fluids and tissues [7].

This article offers an overview of the approaches originally developed in experimental pharmacology by Heinz Otto Schild [9] to characterize and quantify antagonists in animals and organ preparations. The methods aim at establishing agonist dose–effect curves with various doses of antagonists. In the last two decades, they have successfully been extended to apply not only to the renin-angiotensin system but also to clinical pharmacology in general [10, 11], and they have been particularly useful in comparing and characterizing various AT_1 antagonists in man.

Principles of competitive and noncompetitive antagonism and Schild regression techniques

The underlying principles are those of drug–receptor interaction and elicited response such as frequently used in experimental pharmacology [12, 13, 14]. They are based on the assumption that an agonist interacts reversibly with its receptor and consequently induces an effect. The intensity of effect is, though potentially highly nonlinear, related to the number of receptors occupied by the agonist. A maximum effect is achieved when all receptors are theoretically occupied. The observed dose– or concentration–effect relationships are conventionally plotted semilogarithmically, i.e. linear effects on ordinate *vs.* log concentrations on abscissa, and display the typical sigmoid shape. Antagonists bind to the same receptors as the agonists, usually without inducing any effect themselves. Consequently, the agonist molecules are prevented from binding to the receptor and their effect is inhibited. Antagonists are considered competitive if they are bound reversibly; as a result, by increasing the concentration of the agonist, antagonist molecules will be displaced, inhibition overcome, and the same maximum effect obtained as in the absence of the antagonist, but with much higher agonist concentrations. In a graphic plot, the resulting agonist dose–effect curve will appear shifted to the right by the antagonist. The degree of this parallel shift in the lin-log plot depends on the antagonist dose and in turn allows for a quantification of the antagonistic activity. It is expressed as the ratio of the agonist concentrations (or doses) that elicit an identical response both in the presence and the absence of the antagonist and is defined as 'dose ratio' (DR). In experimental pharmacology, DRs are usually derived at agonist concentrations producing half of the maximum effect (E_{50}). In clinical pharmacology, we only obtain a limited segment of the dose–effect curves due to ethical limitations and frequently miss the maximum response (E_{max}). Therefore, DRs in humans are usually derived from data below the EC_{50} [15]. This inevitably means working outside the linear segment of the log concentration–effect curve, but is feasible by means of nonlinear fitting techniques (see Figure 1).

In further analyses, the term DR minus one (DR−1) is used (and not DR) to allow a linear double logarithmic plot *vs.* antagonist concentration. This presents the 'Schild regression plot'. From this plot in experimental and clinical pharmacology, pA_2 concentrations and K_i doses, respectively, are derived as log concentrations and apparent doses, respectively. A detailed example is provided in Figure 2 using data from a clinical study. In *in vitro* experimental pharmacology, DR−1 values are determined by and are directly proportional to the active free concentration in an organ bath. Conversely, DR−1 values

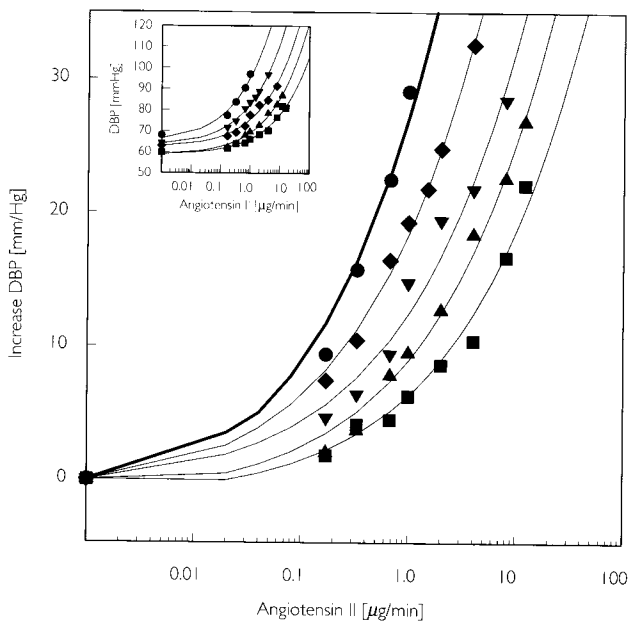

Figure 1 Angiotensin dose–effect in humans. Angiotensin II dose (3 min intravenous infusion per dose step)–effect [increase in diastolic blood pressure (DBP)] curves in healthy humans ($n = 13$). The mean curves before (○) and at various time intervals (●, 0 h (before single dose); ■, 2 h; ▲, 4 h; ▼, 10 h; ◆ 24 h) within 24 h after oral administration of 150 mg of irbesartan were fitted simultaneously (data from [7]).

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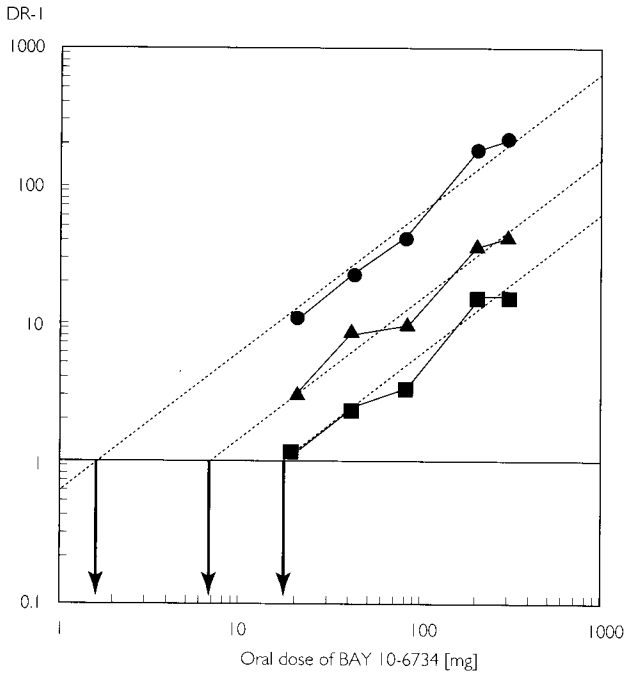

Figure 2 'Schild regression plots' from a clinical study in humans (modified from [21]). In the double logarithmic plot, the DR–1 of the angiotensin II dose–effect curves (diastolic blood pressure) are related to varying doses (20–300 mg) of an oral AT_1 antagonist (BAY 10–6734). Three Schild regression lines were obtained using median data of DR–1 at 2 h (●), 8 h (▲), and 12 h (■) after oral administration. Subsequently, perpendiculars were dropped from the points where the regression lines of the corresponding data points intersect with the DR–1 = 1 line so that the arrows pointing towards the abscissa reveal the 'apparent K_i doses'. K_i doses resemble the pA_2 concentrations of antagonists which in experimental pharmacology are given as the negative logarithm of the molar concentrations at that intersection [14].

represent an *in vivo* bioassay of free active concentration of the parent compound plus active metabolite(s) in the vicinity of the receptor.

When antagonists reduce the maximum agonist effect, the antagonism is considered noncompetitive or unsurmountable. This property can easily be demonstrated in experimental preparations whose concentration ranges are almost limitless. By contrast, conditions in clinical pharmacology only rarely allow for obtaining a maximum agonistic response. Therefore, it is almost impossible to assess whether or not a given antagonism is noncompetitive based on maximum effects.

There is yet another rational approach that permits clinical exploration of this kind of antagonism: the correlation of the antagonist's log concentration (or dose) with the log DR–1 values results in the 'Schild regression plot' (see Figures 2 and 3). Here, a slope of equal to or almost 1 is typical for competitive antagonists. Nonlinearity or a slope significantly different from 1 are indicative of multiple receptors (receptor subtypes) or noncompetitive antagonists [11, 13, 14, 16].

One of the major advantages of the Schild regression plot over other approaches in analysing the antagonist's properties is based on the fact that the results remain unaffected by typical sources of bias such as reflexes, counterregulatory mechanisms, or the physiological response detection method. Even complex multifactorial effects will yield an accurate result in the Schild analysis. Every step(s) between the receptor occupied by the agonist and the effect elicited may be considered a 'black box' basically without relevance to the outcome of the regression plot. The method's only prerequisite is a measurable effect (e.g. increase in heart rate or blood pressure). Under steady-state conditions, the same amount of receptor occupation by agonist molecules will result in the same intensity of effect at points of equal agonist occupancy and irrespective of absence, presence, and concentration of the antagonist.

In terms of the renin-angiotensin system, Schild regression approaches have first been utilized in humans to characterize time kinetics and potency of ACE inhibitors [10, 17]. Angiotensin I served as 'agonist' and a variety of ACE inhibitors as 'antagonists', even though the latter do not antagonize directly at the effector site responsible for the effect measured (i.e. blood pressure increase) but compete with angiotensin I at an earlier reaction step, i.e. at the converting enzyme level. The method permitted differentiation of the duration of action of short and long-acting ACE inhibitors [17] and deriving 'apparent' K_i doses [18] which were closely related to the 'therapeutic' doses.

Angiotensin II dose–effect curves in humans

The Schild method had been initially adopted and described for angiotensin I effects [10]. All studies and methods had been approved by local ethics committees. Starting with a continuous intravenous infusion of 0.33 μg min^{-1}, angiotensin I or II doses are increased stepwise up to a maximum of 20 μg min^{-1} [15]. Each dose level is maintained for 3 min. For safety reasons, blood pressure is monitored in 1-min intervals, and the diastolic blood pressure reached after 3 min at each dose level is used to assess the response. It has been shown that a steady state of response is obtained within 3 min even with Ang I which needs additional time to be converted to Ang II [19]. In contrast to the venous system [20], the pressor response to Ang II did not show any development of tolerance beyond the time needed for the challenges. For safety reasons, diastolic blood pressure of 110 mmHg or a rise of >25 mmHg is the cut-off point for any further dose increase. Observing these safety limitations, we did not detect any relevant

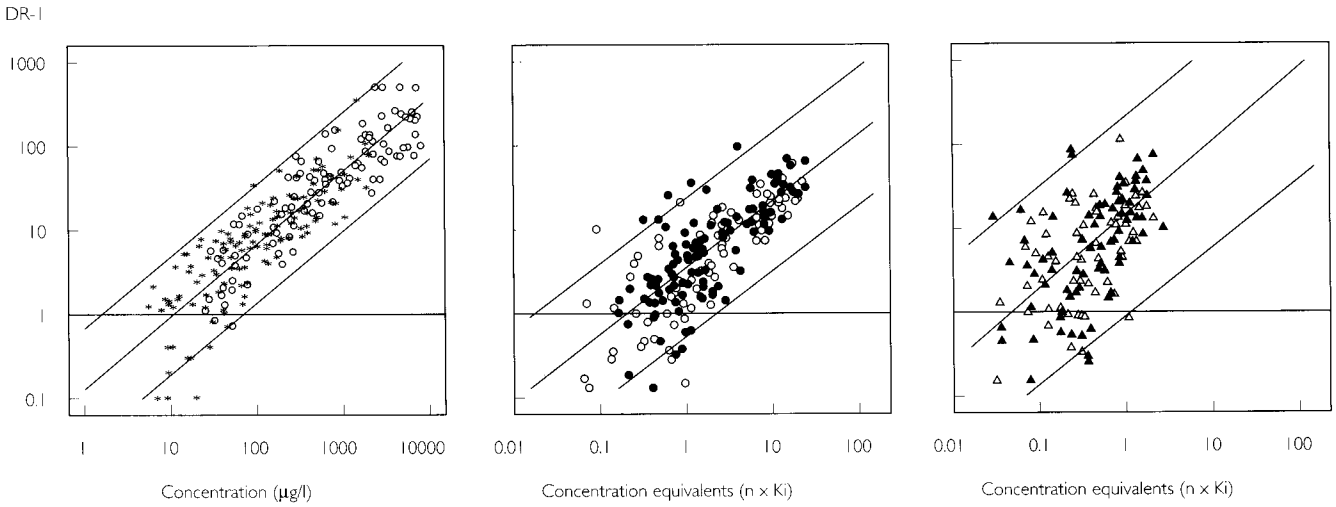

Figure 3 Schild regressions for the competitive AT_1 antagonist BAY 10–6734 (left) and two insurmountable antagonists irbesartan (middle) and candesartan (right) (regression line and 95% confidence intervals depicted). Concentrations for BAY 10–6734 were measured biochemically, for the other substances by radio receptor for ligand binding assay. The AT_1 antagonist Schild regressions reveal linear correlations between the concentration in plasma *ex vivo* and the rightward shift (DR–1) of the Ang II dose–effect curves *in vivo*. Decreasing concentrations of the antagonists were observed for the individual kinetic values during the time of up to 47 h after single (all antagonists) and repetitive (irbesartan and candesartan) doses. The slopes in the double log plots of the three substances are similar and did not significantly differ from 1 (data [7, 21]). Symbols, left plot: *results from 20 to 80 mg; ○, from 200 and 300 mg p.o. of BAY 10–6734; centre and right plots: open symbols results after single, closed symbols after repetitive administration.

problem in over 3000 angiotensin II dose–effect curves derived from healthy human volunteers. As diastolic blood pressure resembles the increase in total peripheral resistance which is close to the underlying angiotensin-induced physiological response, i.e. the arteriolar vasoconstriction, the response of diastolic rather than systolic blood pressure is usually studied [10]. But systolic blood pressure outcomes did not markedly differ from diastolic ones [21]. The resulting initial portions of dose–effect curves, preferably from multiple experiments in the same volunteer, are then repeatedly and simultaneously fitted by nonlinear regression analyses using the sigmoid E_{max} model according to Hill (Figure 1) [15]. E_{max} is arbitrarily set to 500 mmHg to allow for extrapolation of an EC_{50} value. It has been shown that this procedure provides valid and significant DR–1 results [15]. The obtained DR–1 results were then subjected to descriptive and, where indicated, confirmatory standard statistical methods (e.g. see [7, 15, 21]).

Triggering a blood pressure increase by a predefined bolus dose of angiotensin is a frequently used alternative method [22–25]. Compared with the dose–effect procedure as described above, this approach is easier to perform. However, a steady state of angiotensin effect is not reached under these conditions. Consequently, the efficacy of substances with stronger receptor binding will be overestimated. In addition, the method covers only a limited range in which differences in the degree of antagonism can be assessed quantitatively. When the chosen antagonistic dose is high, antagonism seems to reach a ceiling where further dose increases result in no further increase in effect. With respect to time kinetics, the decrease of antagonistic action after high doses appears much slower than that of the corresponding pharmacokinetic properties, and a half-life of effect cannot be quantified. Principles and limitations of these methodologies have been presented for β-adrenoceptor antagonists as a model [26].

Surmountable/insurmountable angiotensin II antagonism in clinical pharmacology studies

Results of experimental pharmacology studies indicate that some of the AT_1 antagonists exert insurmountable effects, i.e. the maximum possible agonistic effect of Ang II is reduced in the presence of the antagonist [27]. The clinical significance of this property has been questioned [6], since the agonist and antagonist levels in humans never reach the high concentrations used in experimental pharmacology and the actions of all these antagonists are basically competitive and reversible in nature [28, 29]. Studies in clinical pharmacology demonstrated that the slopes in Schild plots covering a wide range of concentrations and using the entire scope of clinical doses of agonists and antagonists also revealed slopes that were slightly below 1 and did not differ for competitive AT_1 (BAY 10–6734) or insurmountable AT_1 blockers (irbesartan and candesartan) (Figure 3) [7, 21].

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Irreversible antagonism would have resulted in slopes significantly steeper than 1. Schild regression plots based on different antagonist doses instead of varying concentrations of the antagonists result in similar outcomes, and for most of the available AT_1 antagonists and for BAY 10–6734, the Schild regressions between the orally administered doses and the rightward shift of the Ang II dose–effect curves revealed linear correlations with slopes not significantly different from 1 [21, 30]. These data clearly support the assumption that in the dose ranges used in humans all the substances tested displayed the properties of competitive antagonists and that for the AT_1 antagonists insurmountability is clinically of little or no relevance.

Schild regression analysis for further exploration of the mechanisms of angiotensin II agonism and AT_1 antagonism

Schild regression analyses provide further insights into the nature of the angiotensin II agonism and AT_1 antagonism. The regression plots in Figure 2a–c reveal linear correlations with slopes close to, but slightly below 1 (i.e. 0.85 for Bay 10–6734; 0.84 for candesartan; 0.77 for irbesartan). Whereas slopes steeper than 1 would indicate irreversible antagonism, slopes flatter than 1 are typical for a second mechanism and/or receptor (subtype receptor) [11]. Due to high selectivity, the AT_1 antagonists block AT_1 receptor-mediated agonistic activity of angiotensin II and do not exert any other effects *per se*. In the event of additional phenomena, the agonist should be considered the cause. It had been shown in humans that Ang II infusion increases concentrations of the vasodilatory NO in plasma [31]. As this occurred equally in the absence and presence of AT_1 blockade, it pointed to an additional, non-AT_1 receptor-mediated effect of Ang II *in vivo*. This mechanism could attenuate or (under AT_1 blockade) even reverse the vasoconstrictor response of Ang II [31]. Such a normally hidden mechanism appears to have been disclosed in our studies with the infusions of higher Ang II doses; they had been administered at high antagonist concentrations/doses to induce a blood pressor response in the presence of AT_1 receptor blockade. Vasodilatory counterregulation due to Ang II may cause DR–1 to be lower than expected at increasing concentrations of antagonists and may result in the observed slopes lower than 1 in Schild plots. The assumption that a second, usually hidden, counterregulatory vasodilatory Ang II mechanism of action becomes overt with AT_1 blockade is further supported by a correlation between increase in plasma renin activity and lowering of diastolic blood pressure with AT_1 blockade which cannot be explained by the correlation between renin and vascular AT_1 antagonism [7]. Thus the question arises as to which basic physiological mechanism(s) may cause such a vasodilatory effect of Ang II. As currently discussed, vasodilation may be explained by the stimulation of AT_2 receptors [32], or, alternately, the metabolite Ang1–7 [33], since formation of this metabolite will increase with higher concentrations of Ang II. In addition, interactions between AT_1 blockers and prostaglandin systems are currently under discussion.

In summary, various observations suggest that beyond the antipressor effects [34] mediated via AT_1 blockade, additional effects are present which could in part be explained by a second normally hidden mechanism of action of angiotensin II.

Potency of AT_1 receptor antagonists

The Schild regression technique allows one to derive apparent K_i doses, i.e. the antagonist doses producing a DR–1 of 1, or a twofold rightward shift of the Ang II dose–effect curve [21, 30]. Apparent K_i doses may be used to compare the antagonistic potency of various substances on a rational basis. In a recent study comparing the potency of several AT_1 antagonists, candesartan clearly exhibited the highest activity per mg of substance identified by the lowest apparent K_i dose after oral administration [30]. It is important to keep in mind that apparent K_i doses are time dependent, i.e. the greater the time gap between administration and measurement and the shorter the half-life, the greater the obtained apparent K_i dose. This time dependency can be assessed quantitatively as 'doubling' time, i.e. the time necessary for doubling the apparent K_i dose. The doubling time may be viewed as the inverse of the half-life time [21]. Apparent K_i doses of AT_1 antagonists at 24 h average the dose ranges which have to be administered to obtain a 24-h effect [30].

Kinetics of DR–1

A major application of the method titrating AT_1 blockade is the derivation of time kinetics of antagonism [35]. Figure 4 shows an example of the log linear decline of DR–1 following administration of four AT_1 antagonists. The markedly slower fall of direct pressor antagonistic effects following candesartan and irbesartan can be differentiated from the more rapid decay following losartan and valsartan. These findings are in accordance with pharmacokinetic results, while the half-lives derived from the two different approaches are almost identical (Table 1). In the case of active/inactive metabolites such as losartan, the DR–1 method presumably detects the

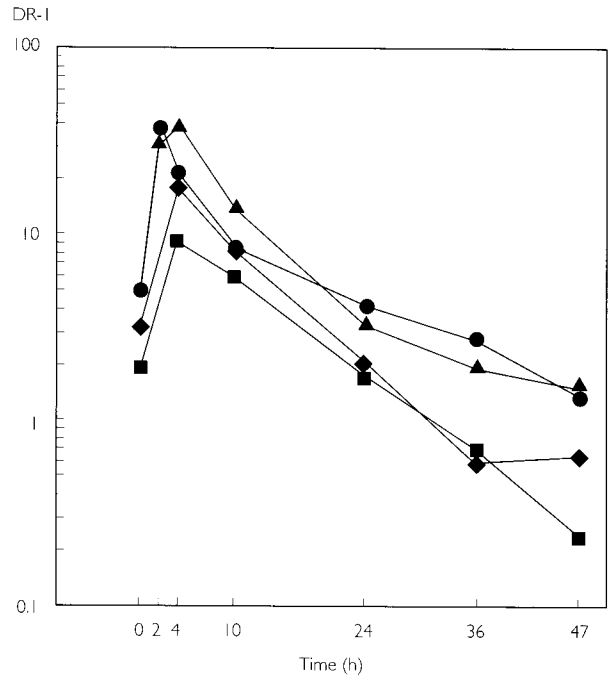

Figure 4 Log-linear plot of the time course of mean DR–1 values before (0 h) and up to 47 h after the last dose of a 6-day administration of irbesartan (●, 150 mg day^{-1}), valsartan (◆, 80 mg day^{-1}), losartan (■, 50 mg day^{-1}), and candesartan (▲, 8 mg day^{-1}) (adopted from [7, 34]).

Table 1 Half-lives of various AT_1 antagonists in pharmaco-kinetic and -dynamic (DR–1) measurements (data from [6–8, 34]).

	Pharmacokinetic (chemical) $t_{1/2}$ *(h)*	*DR–1 measurement* $t_{1/2}$ *(h)*
Candesartan	9	12
Irbesartan	11–15	15–18
Losartan (Exp. 3174)	2 (6–9)	(8)
Valsartan	6–9	8

For losartan, the dynamic half-life corresponds to the results of the active metabolite.

'real' biological activity and its decay, independent of whether metabolites and/or the parent compound trigger this activity.

DR–1 as a bioassay of active concentration at receptor site

The time kinetics of DR–1 offers an additional opportunity to compare pharmacological properties of the AT_1 blockers in humans: since DR–1 is an independent entity and a commensurable bioassay of active free antagonistic concentrations around the receptor, it may be utilized to demonstrate concentration–effect relationships. In single

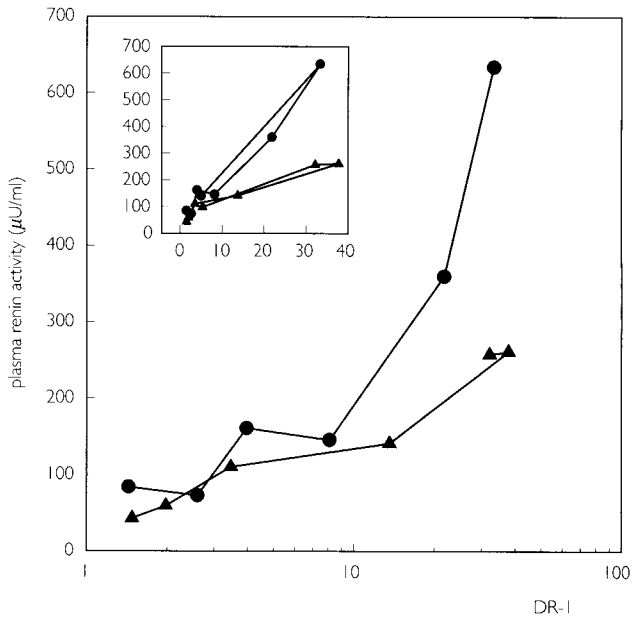

Figure 5 Concentration equivalents assessed by DR–1 at the receptor site in humans after 1 week's administration of Ang II dose–response *vs.* pharmacological effect as plasma renin increase of the two angiotensin AT_1 receptor antagonists irbesartan (●) and candesartan (▲). The inset provides the underlying time-dependent relations between DR–1 and plasma renin activity presented as almost closed narrow hysteresis loops over time (data adopted from [7]). Note, the concentration equivalent dose–effect curves in humans will in most instances, due to ethical limitations in experimental studies in humans, preclude assessment of the complete dose–effect curves.

dose–level studies, an adequate approach based on the fall in DR–1 used as concentration equivalents has been adopted from the time–dose–response design of Tallarida *et al.* [36]. In a recently published study, this method allowed us to detect pharmacodynamic differences between two AT_1 antagonists [7], as demonstrated in Figure 5. The log DR–1 is depicted as antagonistic concentration equivalents on the abscissa; the renal effect of AT_1 antagonists assessed by increase in plasma renin activity is shown on the ordinate. It becomes apparent that at lower concentrations – up to DR–1 of 10 – the two antagonists are indistinguishable. At higher concentrations, the two antagonists behave differently and, though only a segment of the dose–effect curve was obtained, the potency of irbesartan to increase plasma renin levels is at least twice that of candesartan.

When using this approach, it must be kept in mind that possible compartmentalization and an open hysteresis loop would require pharmaco-kinetic and -dynamic modelling [37, 38].

The methodology clearly diverges from conventional hypertension studies which are not suited to differentiate

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between various compounds. Since the primary target in the clinical development of these substances was the lowering of blood pressure, they indeed showed an equivalent blood pressure-lowering action [39]. Pharmacodynamic comparisons of a broader range of effects should not be based solely on a relatively insensitive method with the limited scope of blood pressure-lowering effect. Rather, they require methods that accurately measure degree, time kinetics, and nature of the antagonism in humans and thus provide a quantitative data base.

This review draws on the work of cooperating colleagues and I am most grateful for the invaluable contributions of Dr Raunhild Butzer and Dr Kerstin Breithaupt-Grögler (ZeKaPha, Mainz, Germany), as well as of Professor Dr Anton Wellstein (Georgetown University, Washington, DC, USA). For the many stimulating discussions, the author is deeply indebted to Professor Dr Dieter Palm, Emeritus Director of the Pharmacologic Institute of the University of Frankfurt, Germany.

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