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Pharmacological profile of GR117289 *in vitro*: a novel, potent and specific non-peptide angiotensin AT_1 receptor antagonist

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1 This paper describes the effects of GR117289 (1-[[3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl]methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid) at angiotensin receptors and binding sites in rabbit aorta, rat liver and bovine cerebellum preparations *in vitro*.

2 In rabbit isolated aortic strips, GR117289 (0.3, 1 and 3 nM) caused a concentration-related, insurmountable suppression of the concentration-response curve to angiotensin II (AII). When the contact time was increased, a greater degree of antagonism of AII was observed, suggesting that GR117289 is slow to reach equilibrium. A pK_B of 9.8 ± 0.1 was calculated for GR117289 after 3 h incubation. GR117289 (1 μM) did not affect contractile responses to phenylephrine or 5-hydroxytryptamine (5-HT) in the rabbit aorta.

3 GR117289 (1 nM) alone caused a marked suppression and a slight rightward displacement of the AII concentration-response curve. Co-incubation with the competitive, surmountable AT_1 receptor antagonist, losartan (10 nM, 100 nM and 1 μM), resulted in a concentration-related upward and rightward displacement of the concentration-response curve to subsequently administered AII. In separate experiments in which preparations were pre-incubated with GR117289 (1 nM), subsequent addition of losartan (1 μM) for 2, 15 or 45 min caused a further, but similar, rightward displacement of the concentration-response curve to subsequently administered AII with a *time-dependent increase* in the maximum response.

4 Suppression of AII-induced contractile responses, caused by superfusion with GR117289 (0.3, 1 or 3 nM) was not reversed by continuously washing the tissues for 3 h; in fact, the potency of GR117289 was slightly enhanced after this period.

5 In rat liver membranes, GR117289 was a potent competitor with [^{3}H]-AII for AT_1 binding sites ($pK_i = 8.7 \pm 0.1$) but in bovine cerebellum membranes, it was a very weak competitor for AT_2 binding sites ($pK_i < 6$). Pre-incubation of rat liver membranes with GR117289 had little effect on its affinity ($pK_i = 9.1 \pm 0.21$), but increasing the concentration of bovine serum albumen in the assay buffer from 0.001% to 0.1% w/v decreased affinity ($pK_i = 7.5 \pm 0.1$).

6 In saturation binding experiments in rat liver membranes, GR117289 (12 nM) increased the K_d of [^{3}H]-AII from 0.28 ± 0.06 nM to 0.37 ± 0.02 nM, and decreased B_{max} from 10.0 ± 0.1 to 5.6 ± 0.3 fmol mg^{-1} tissue. In other experiments, GR117289 (1 μM) did not alter the rate of dissociation of [^{3}H]-AII from AT_1 binding sites, following addition of excess unlabelled AII.

7 In rabbit aorta vascular smooth muscle membranes, GR117289 competed with [^{125}I]-Sar^{1}Ile^8 AII for binding to AT_1 binding sites. In the presence of 0.1% w/v bovine serum albumen, a pIC_{50} of 7.6 ± 0.1 was calculated. Under the same conditions, but with rat liver membranes, a pIC_{50} of 7.8 ± 0.1 was determined.

8 Taken together, these results show that GR117289 is a potent, specific, selective and insurmountable antagonist at angiotensin AT_1 receptors. Its profile in the rabbit aorta is consistent with the proposal that GR117289 is a slowly reversible (pseudo-irreversible) antagonist at these receptors.

Keywords: GR117289; angiotensin II (AII); AT_1 and AT_2 receptors; rabbit aorta; rat liver; bovine cerebellum; insurmountable antagonist

Introduction

The renin-angiotensin-aldosterone system plays a pivotal role in cardiovascular homeostasis, and the therapeutic success of angiotensin converting enzyme (ACE) inhibitors, such as captopril and enalapril, in the treatment of hypertension and heart failure has confirmed the involvement of this system in these disease states. Despite their clinical success, the use of ACE inhibitors is not without problems, dry cough and a propensity to induce functional renal failure being amongst the most common (Gavras & Gavras, 1988).

An alternative way of intervening in the renin-angiotensin-aldostererone axis, that might avoid the side effects of ACE inhibitors, is to prevent the action of the main effector of this system, angiotensin II (AII), at its sites of action. Until recently, angiotensin receptor antagonists have been peptidic in nature, and many have retained significant agonist activity. This, coupled with their short duration of action when administered systemically, and poor oral bioavailability, has prevented these compounds from establishing a therapeutic role. However, in 1988, Timmermans and associates reported that a series of 1-benzylimidazole-5-acetate derivatives exhibited significant affinity for angiotensin receptors in isolated tissues, including rat adrenal cortical microsomes and rabbit isolated aorta (Chiu *et al.*, 1988; 1989). The best known example of these compounds is losartan (DuP753;

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Chiu et al., 1990). However, comparison of the profiles of action of compounds such as losartan, with that shown by imidazopyridines such as PD123177 (see Wong et al., 1990a), has revealed the existence of (at least) two distinct populations of angiotensin binding sites. These two binding sites are generally assumed to be synonymous with different angiotensin receptors, and it has been proposed that these be termed AT_1 and AT_2 receptors (Bumpus et al., 1991). Losartan and PD123177 are archetypal AT_1 and AT_2 receptor antagonists, respectively. The AT_1 receptors appear to mediate most, if not all, of the established effects of AII (e.g. vasoconstriction, steroidogenesis, dipsogenesis) and the role, if any, of AT_2 receptors remains unclear (e.g. see Zarahn et al., 1992).

Recently, Middlemiss et al. (1991) reported on a series of bromobenzofurans, a novel class of potent, non-peptide, angiotensin receptor antagonists. These compounds inhibit AII-induced contraction of rabbit isolated aortic strips and reduce blood pressure in renal artery-ligated, hypertensive rats. Among these compounds, GR117289 (1-[[3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl]methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid) (Figure 1) exhibited potent AII antagonist activity *in vitro*, and exerted marked and prolonged antihypertensive activity after oral administration *in vivo*. This paper presents a detailed account of the pharmacological profile of GR117289 *in vitro*. Preliminary accounts have been presented to the British Pharmacological Society (Marshall et al., 1991; Robertson et al., 1991).

Methods

Functional studies in rabbit aorta

A detailed account of the rabbit isolated aortic strip preparation, and the experimental protocol used have been given previously (Robertson et al., 1992). Briefly, helical strips (1.5–2 cm) of thoracic aorta from male, New Zealand White rabbits were suspended in glass tissue chambers under a resting tension of 0.5 g in a physiological salt solution at 37°C, containing indomethacin (30 μM) and ascorbic acid (100 μM), and gassed with 95% O_2/5% CO_2.

In each experiment, four strips of aorta from a single rabbit were used simultaneously. In one series of experiments, the tissues were suspended under a resting tension of 0.5 g in a superfusion apparatus (see Coleman & Nials, 1989) in which the preparations were constantly superfused with physiological salt solution at 37°C.

Experimental protocols

In 4 separate, matched preparations, three consecutive concentration-contractile response curves to AII (0.1 nM–100 nM) were constructed until reproducible, by the cumulative addition of AII to the bathing fluid. The last of these was termed the 'pretest' curve. Subsequently, three tissues were exposed to one of three concentrations of GR117289 (0.3, 1 or 3 nM), for periods ranging from 45 min to 4 h. A fourth tissue was exposed to vehicle alone and served as a time-matched control. After the antagonist (or vehicle) incubation period, a final ('test') cumulative AII concentration-response curve was constructed. In a separate series of experiments, losartan (10, 100 or 1000 nM) was co-incubated for varying periods with GR117289 (1 nM) before the test AII curve. In some experiments, 5-hydroxytryptamine (5-HT, 30 nM–10 μM) or phenylephrine (10 nM–30 μM) was used in place of AII throughout.

In experiments using superfusion, cumulative AII concentration-response curves were constructed by sequentially increasing the concentration of AII in the superfusate. The flow rate over each tissue was 2 ml min^{-1}. AII was introduced into the superfusate in solution at a rate of 0.02 ml min^{-1}. GR117289 (0.3, 1 or 3 nM), or vehicle, was superfused for 45 min over the tissues (again, the flow rate of solution containing GR117289 was 0.02 ml min^{-1}). A final AII concentration-response curve (test) was then constructed, either immediately following the GR117289 (or vehicle) contact period, or after a subsequent 3 h period during which time the tissues were superfused (washed) with physiological salt solution alone.

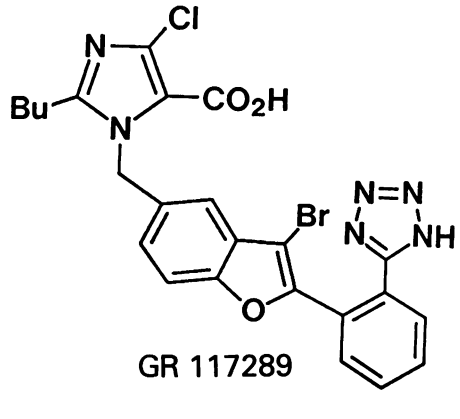

Figure 1 Chemical structure of GR117289.

Expression of results: determination of antagonist potency

For any individual preparation, the contractile response to each concentration of AII (or 5-HT, or phenylephrine) in the test curve was expressed as a percentage of the maximum contractile response to the agonist in the pretest curve. Values are shown as mean ± s.e.mean. In some cases, the concentration of agonist required to elicit a half maximum response (EC_{50}) was calculated; geometric mean (95% confidence intervals) EC_{50} values were calculated from these data.

Incubation with GR117289 reduced the maximal AII contractile response (insurmountable antagonism). For this reason, conventional Schild analysis could not be used to obtain a measure of potency of GR117289. Instead, an apparent pK_B for GR117289 was derived by use of a double-reciprocal regression plot (Kenakin, 1984). A graph of 1/A vs 1/A' was plotted, where A and A' were the equieffective concentrations of AII in the absence or presence of GR117289, respectively. The gradient (G) of this plot was then used in the Gaddum equation to obtain an estimate of pK_B: thus, $pK_B = -\log([B]/G-1)$, where B is the antagonist concentration.

Radioligand binding studies

Livers were obtained from Lister hooded rats (300–400 g) which were killed by cervical dislocation, and aortic tissue was obtained from male New Zealand White rabbits. Bovine cerebellum was obtained from Glaxo Institute for Molecular Biology, Geneva. The tissue was homogenized in 10–20 volumes (w/v) of ice cold homogenizing buffer (50 mM Tris, 5 mM EDTA, pH 7.4 at 4°C) in a Polytron P10 homogenizer. The homogenate was centrifuged at 48,000 *g* for 12 min at 4°C and the supernatant discarded. The pellet was resuspended in homogenizing buffer using the Polytron P10 and then centrifuged as before. The final pellet was resuspended in 50 mM Tris buffer (pH 7.4 at 25°C) at a tissue concentration of 400 mg ml^{-1} and stored at −70°C until required.

[^{3}H]-angiotensin II binding to rat liver and bovine cerebellum membranes

Binding assays were performed by incubating the membranes (5 mg tissue/tube) in 500 μl of assay buffer (Tris 50 mM,

NaCl 100 mM, $MgCl_2$ 10 mM, EDTA 1 mM, BSA 0.001% w/v and bacitracin 1 mM) with approximately 0.3 nM [^{3}H]-AII with or without competing compounds. Assay tubes were incubated at room temperature for 90 min, after which time, bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass fibre filters, which were pretreated with 0.1% polyethyleneimine (PEI), using a Brandel cell harvester. The filters were washed with ice cold wash buffer (100 mM NaCl, 5 mM $MgCl_2$) and trapped radioactivity was determined by liquid scintillation counting using a Packard 2200Ca scintillation counter. Specific binding was defined as that displaceable by 1 μM AII. In saturation experiments, rat liver membranes were incubated with increasing concentrations of [^{3}H]-AII as described. Bound radioactivity, in the presence and absence of 1 μM AII for each concentration of [^{3}H]-AII, was determined by filtration as described above. To determine the effects of GR117289 on saturation binding, membranes were incubated with 12 nM GR117289 for 30 min prior to the addition of the [^{3}H]-AII. In dissociation experiments, [^{3}H]-AII (0.3 nM) was incubated to steady state with rat liver membranes (45 min at room temperature) in magnesium-free assay buffer. AII (1 μM), with or without GR117289 (final concentration, 1 μM), was then added and the amount bound after various time intervals between 0 and 240 min was determined by filtration as previously described. Membranes were incubated to steady state in the presence (total) or absence (nonspecific) of 1 μM AII and the dissociation rates of specific binding (total minus nonspecific binding) were determined.

[^{125}I]-Sar^1-Ile^8-angiotensin II binding

Membranes (0.1 mg tissue/tube for rat liver and 10 mg tissue/tube for rabbit aorta) were incubated in 150 μl of assay buffer (Tris 50 mM, NaCl 100 nM, $MgCl_2$ 10 mM, EDTA 1 mM, BSA 0.1% w/v and bacitracin 1 mM) with approximately 0.05 nM [^{125}I]-Sar^1-Ile^8-angiotensin II ([^{125}I]-Sarile) with or without competing compounds. Assay tubes were incubated for 90 min at room temperature after which time, bound and free radioactivity was separated as described above. Trapped radioactivity was measured with an LKB 1282 Compugamma counter. Specific binding was defined as that displaceable by 3 μM AII.

Data analysis

Competition binding data were analysed by use of iterative curve fitting techniques (Michel & Whiting, 1984). Data from [^{125}I]-Sarile binding experiments are presented as the negative logarithm of the IC_{50} (pIC_{50}). In the case of [^{3}H]-AII experiments, the IC_{50} values were corrected for the presence of the radioligand using the Cheng-Prusoff approximation (Cheng & Prusoff, 1973) and are presented as pK_i. Saturation experiments are presented in a Scatchard plot. K_d and B_{max} values were calculated by use of LIGAND (Munson & Rodbard, 1980). In dissociation experiments, dissociation rates were calculated using ENZFITTER (Biosoft, Cambridge).

Drugs used

Angiotensin II (human sequence) was obtained from Nova biochem., U.K. Ltd., Bacitracin, bovine serum albumin (BSA), 5-hydroxytryptamine (creatinine sulphate complex), phenylephrine hydrochloride and indomethacin were obtained from Sigma. [^{3}H]-angiotensin II ([^{3}H]-AII) and [^{125}I]-Sar^{1}Ile^8 angiotensin II ([^{125}I]-Sarile) were obtained from NEN, Du Pont (specific activities of 73.4 and 2200 Ci mMol^{-1}). GR117289 and losartan (potassium salt) were synthesized in the Chemistry Division, Glaxo Group Research Ltd.

Angiotensin II was dissolved in distilled water and stored frozen as aliquots (1 mM) which were thawed on the day of experimentation and diluted with distilled water. Phenylephrine and 5-HT were dissolved and diluted with distilled water. Indomethacin was dissolved in NaOH (2 M) and added directly to the physiological salt solution to give a final concentration of 30 μM. Bacitracin and BSA were dissolved directly in Tris buffer.

All other chemicals and constituents for the physiological salt solution were of Analar grade and obtained from BDH Ltd., England.

Results

The effect of GR117289 on angiotensin II-induced contraction in rabbit aortic strips

Cumulative addition of AII (0.3 nM–0.3 μM) caused concentration-related contractions of rabbit isolated aortic strips. After a 45 min incubation period, GR117289 (0.3, 1 or 3 nM) produced a concentration-related, insurmountable antagonism of AII (Figure 2a). GR117289 (3 nM) caused approximately 65% suppression of the maximum response to AII. Despite this profound, concentration-related suppression of the maximum response to AII, GR117289 did not significantly change the EC_{50} of AII. The geometric mean EC_{50} (95% confidence limits) values in the absence and presence of 0.3, 1 or 3 nM GR117289 were 5.4 (3.4–8.7), 5.3 (3.2–8.3), 5.85 (4.7–7.1), and 6.13 (5.5–6.8)nM, respectively.

For comparison, Figure 2b shows the effect of the sur-

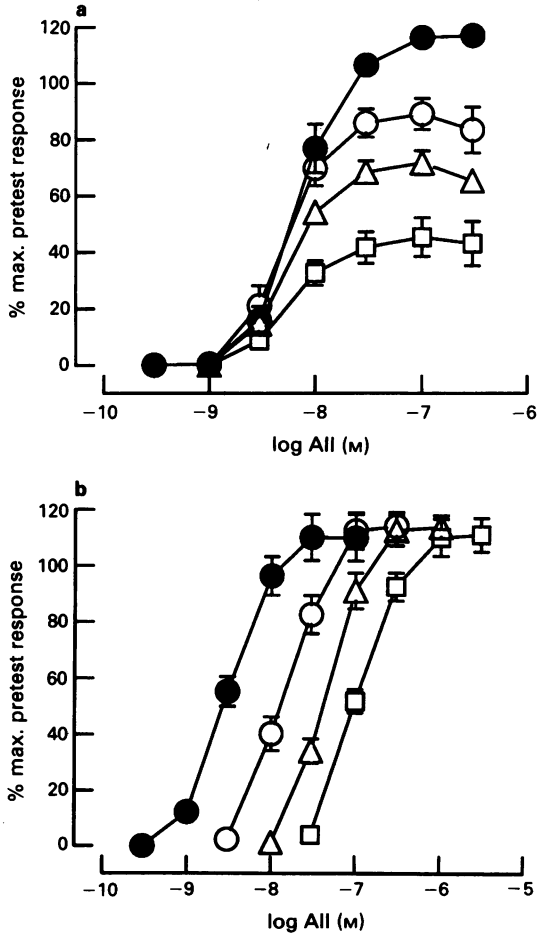

Figure 2 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the presence of (a) GR117289 (○ 0.3, △ 1 or □ 3 nM) or (b) losartan (○ 30, △ 100 or □ 1000 nM) or vehicle for losartan or GR117289 (●) previously incubated for 45 min ($n = 4$ for each). Results are shown as mean response (± s.e.mean, vertical bars) in the test curve, expressed as a % of the maximum response to AII in the pretest curve. Data for losartan are from Robertson *et al.* (1992) and republished with permission.

mountable, competitive angiotensin receptor antagonist, losartan (30, 100 and 300 nM) on AII-induced contractions in the rabbit aorta: losartan did not affect the maximum response to AII. As found previously (Robertson *et al.*, 1992), the pA_2 of losartan was calculated to be 8.2 ± 0.1 (slope = 1.1 ± 0.1) by Schild analysis.

Like AII, both 5-HT (30 nM–10 μM) and phenylephrine (10 nM–30 μM) caused concentration-related contractions in the rabbit aorta. GR117289 (1 μM) had no significant effect on the contractile response to either agonist (data not illustrated).

AII concentration-response curves, before and after a 3 h incubation with the vehicle for GR117289 (pretest and test curves) were highly reproducible. Compared with their respective time-matched vehicle controls, a 3 h incubation with GR117289 (0.3 nM) caused a more profound reduction (77% suppression, Figure 3a) of the maximum response to AII than had been observed after only 45 min (25% suppression, Figure 2a) or 2 h incubation (36% suppression, data not shown). In tissues incubated with vehicle for 4 h, AII responses became more variable (Figure 3b). However, GR117289 (0.3 nM), caused little further reduction (81% suppression, Figure 3b) of the maximum response to AII under these conditions, compared with that seen after 3 h incubation. Thus, GR117289 seemed to have reached equilibrium after approximately 3 h incubation. Using the method of Kenakin (1984), the pK_B of GR117289 was derived from 7 separate experiments in which GR117289 (0.3 nM) was incubated with aortic tissue for 3 h: a pK_B of 9.8 ± 0.1 was calculated. The geometric mean EC_{50} (95% confidence interval) of AII was significantly greater ($P < 0.005$, Student's unpaired t test) in the presence of GR117289 (8.2 (5.1–13.2) nM), than in its absence (2.7 (1.5–4.9) nM), indicating a small rightward displacement of the AII concentration-response curve.

The effect of losartan on GR117289-induced antagonism of angiotensin II

In tissues incubated with GR117289 (1 nM) for 2 h 45 min, the AII concentration-response curve was markedly suppressed and displaced to the right, compared with time-matched, vehicle-treated controls. In preparations which were co-incubated with losartan (10 nM, 100 nM or 1 μM) for the final 45 min of the GR117289 incubation period, the subsequent AII concentration-response curves were displaced upwards (with 10 nM, 100 nM or 1 μM losartan) and to the right (with 100 nM or 1 μM losartan), in a concentration-related manner (Figure 4).

In a related series of experiments, GR117289 (1 nM) incubated with rabbit aorta for 2 h, again caused a marked suppression and rightward displacement of the AII concentration-response curve, although this was not as marked as that seen when the incubation time was 2 h 45 min (compare Figure 5 with Figure 4). In the same series of experiments, GR117289 (1 nM) was incubated for 2 h and then losartan (1 μM) was also added for either 2, 15 or 45 min, before construction of the test AII curve. Co-incubation of losartan with GR117289 caused a larger rightward displacement of the AII concentration-response curve

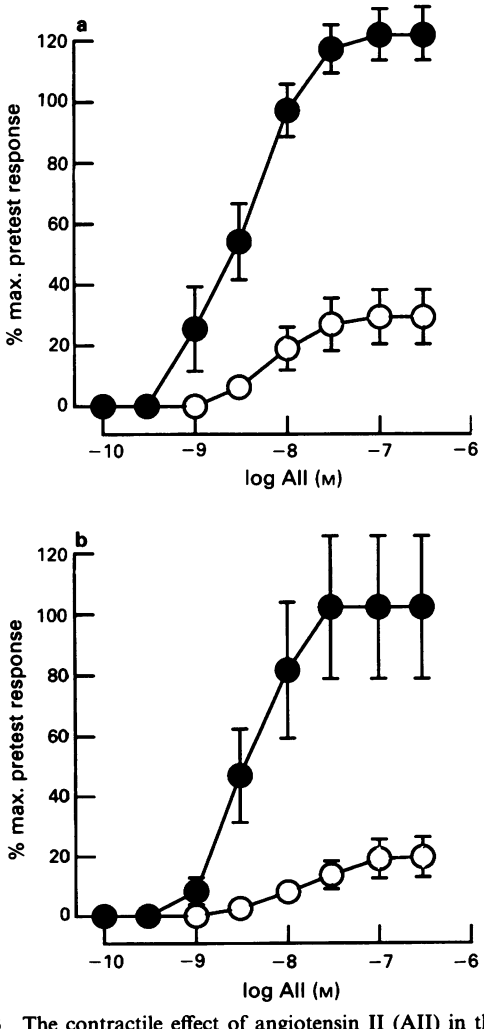

Figure 3 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the presence of GR117289 (○ 0.3 nM) or vehicle for GR117289 (●) incubated for either (a), 3 h or (b), 4 h ($n = 4$ for each).

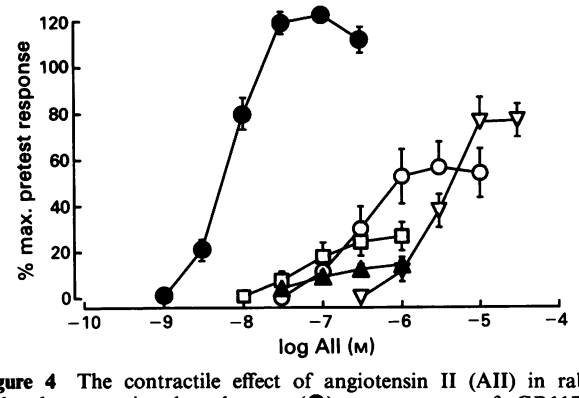

Figure 4 The contractile effect of angiotensin II (AII) in rabbit isolated aorta, in the absence (●) or presence of GR117289 (▲ 1 nM, 2 h 45 min incubation) alone, or losartan (□ 10 nM, ○ 100 nM, or ▽ 1 μM) co-incubated with GR117289 for the final 45 min of the 2 h 45 min incubation period ($n = 4$).

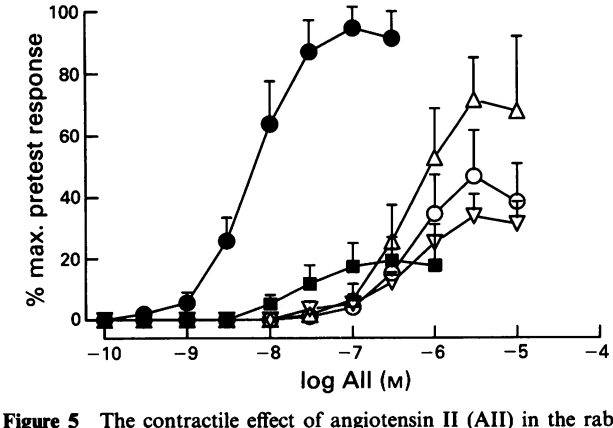

Figure 5 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the absence (●) or presence of GR117289 (■ 1 nM, 2 h incubation) alone, or co-incubated with losartan (1 μM) for a further 2 min (▽), 15 min (○) or 45 min (△) ($n = 5$).

than incubation with GR117289 alone. The concentrations of AII required to produce 50% of the maximum response in the test curve, in preparations co-incubated with losartan and GR117289 were similar, regardless of the duration of the losartan incubation (geometric mean EC_{50} values (95% confidence limits) after 2, 15 and 45 min losartan co-incubation were 731 (224–2381), 621 (260–1483) and 625 (209–1875)nM, respectively). In contrast, co-incubation with losartan caused a time-related increase of the maximum response to AII compared with that observed in preparations treated with GR117289 alone (Figure 5).

The effect of continuous washing on GR117289 antagonism of angiotensin II in superfused rabbit aortic strips

In superfused rabbit aortic strips, increasing concentrations of AII (1–30 nM) caused concentration-related increases in the contractile tension. The sensitivity of the preparations to AII was similar to that seen in the experiments carried out in a fixed volume of bathing fluid. As before, GR117289 (0.3, 1 or 3 nM) perfused over the tissues for 45 min, caused a concentration-related, insurmountable suppression of the contractile response to AII. The highest concentration of GR117289 used (3 nM), reduced the maximum response to AII by approximately 92%. When separate tissues from the same rabbits were treated in the same way with GR117289, but then superfused with drug-free physiological salt solution alone for another 3 h, subsequent AII responses were not restored; rather they were reduced further (see Figure 6b).

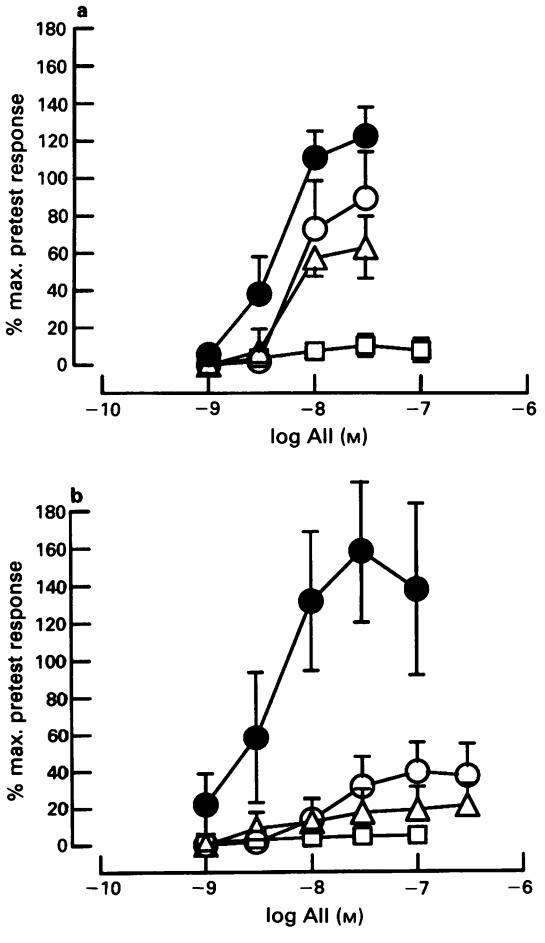

Figure 6 The contractile effect of angiotensin II (AII) in rabbit superfused isolated aorta, (a) in the absence of GR117289 (●) or after GR117289 (○ 0.3, △ 1 or □ 3 nM) was superfused for 45 min or, (b) in the absence of GR117289 (●) or after GR117289 (○ 0.3, △ 1 or □ 3 nM) were superfused for 45 min and followed by drug-free physiological salt solution (prolonged washing) for 3 h, respectively ($n = 3$).

Characteristics of GR117289 binding in rat liver and bovine cerebellum

In competition binding studies using [^{3}H]-AII at angiotensin AT_1 receptors in rat liver membranes, GR117289 was potent with a pK_i of 8.7 ± 0.1 ($n = 12$) and a Hill coefficient (nH) close to unity (1.11). In contrast, in bovine cerebellum, GR117289 had negligible affinity for angiotensin AT_2 sites ($pK_i < 6$, $n = 2$).

The AT_1 receptor has been shown to exist in high and low affinity states depending on its G-protein coupling. The competition studies described above were conducted in the presence of Mg^{2+}, which would result in the majority of the receptors being in the high affinity state with respect to agonist binding. In order to determine whether GR117289 could discriminate between high and low affinity states of the AT_1 receptor, competition studies were carried out in the presence and absence of Mg^{2+}. Under these conditions, the pIC_{50} values were found to be 8.5 ± 0.1 ($n = 3$, nH = 0.99) and 8.7 ± 0.2 ($n = 3$, nH = 1.31), respectively.

In order to determine whether GR117289 attained equilibrium during the 90 min incubation period of the [^{3}H]-AII binding assay, the liver membranes were preincubated with the antagonist for 1 h before addition of the radioligand. A 1 h preincubation resulted in a small, but not significant, increase in the pK_i from 8.7 ± 0.3 ($n = 3$, nH = 1.06) to 9.1 ± 0.21 ($n = 3$, nH = 1.4).

To establish the nature of the antagonism exerted by GR117289, its effect on the saturation curve of [^{3}H]-AII binding was determined. The results for GR117289 (12 nM) are shown in Figure 7 in the form of a Scatchard analysis. GR117289 (12 nM) decreased the B_{max} from 10.0 ± 0.1 fmol mg^{-1} tissue to 5.6 ± 0.3 fmol mg^{-1} tissue and increased the K_d of [^{3}H]-AII-binding from 0.28 ± 0.06 nM to 0.37 ± 0.02 nM ($n = 3$).

In order to investigate further the nature of the antagonism, the effect of GR117289 (1.0 μM) on the dissociation of [^{3}H]-AII, caused by addition of an excess of unlabelled AII, was measured. There was no significant difference between the dissociation rate in the absence ($t_{\frac{1}{2}} = 6.5 \pm 1.1$ min) or presence ($t_{\frac{1}{2}} = 7.4 \pm 1.9$ min) of GR117289 (1.0 μM, $n = 3$).

The affinity of GR117289 in binding assays was found to be markedly dependent on the concentration of BSA in the assay buffer. Increasing the BSA from 0.001% to 0.1% w/v resulted in a large rightward displacement of the inhibition curve which described the competition of [^{3}H]-AII with GR117289. In the presence of 0.1% w/v BSA, the pK_i of GR117289 in the rat liver decreased to 7.5 ± 0.1 (nH = 1.11, $n = 5$).

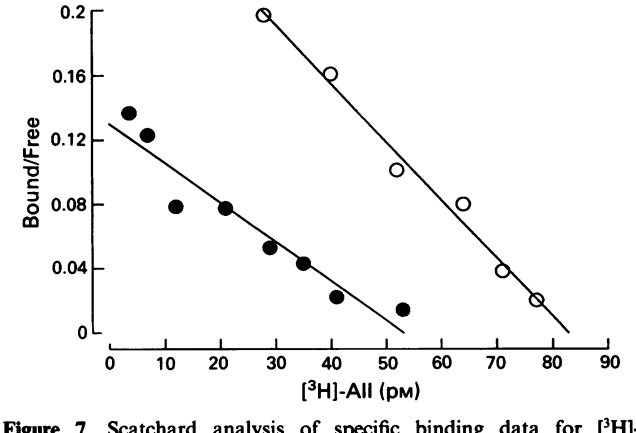

Figure 7 Scatchard analysis of specific binding data for [^{3}H]-angiotensin II ([^{3}H]-AII) in the absence (○) or presence (●) of GR117289 (12 nM). Data are taken from a representative experiment.

Characteristics of GR117289 binding in rabbit aorta

In an attempt to correlate findings in functional and radioligand binding sites, the affinity of GR117289 for angiotensin binding sites in rabbit aorta smooth muscle membranes was determined. Because of the low density of receptors in this tissue, a higher specific activity ligand, [^{125}I]-Sarile was used. In addition, to increase specific binding, the assay buffer contained 0.1% w/v BSA. Under these conditions, GR117289 competed with [^{125}I]-Sarile for binding to angiotensin receptors and yielded a mean pIC_{50} of 7.6 ± 0.1 (nH = 1.13, $n = 3$). Under the same conditions, but with rat liver membranes, the pIC_{50} for GR117289 was 7.8 ± 0.1 (nH = 1.08, $n = 5$).

Discussion

GR117289 is a potent antagonist against AII in rabbit aortic strips, and competes with [^{3}H]-AII for binding sites in rat liver and with [^{3}H]-AII or [^{125}I]-Sarile on rabbit aorta vascular smooth muscle membranes. These preparations were chosen because it is well established that the angiotensin receptors present belong to the AT_1 subtype (Wong *et al.*, 1990a; de Gasparo *et al.*, 1990; Chang & Lotti, 1991) and AT_1 receptors are the only subtype that, thus far, are known to mediate the major physiological effects of AII. In contrast, GR117289 had negligible affinity for AT_2 sites in bovine cerebellum, thus establishing its high selectivity for AT_1 receptors.

We have previously confirmed functionally that losartan is a potent, competitive, surmountable antagonist ($pA_2 = 8.2$) of AII in the rabbit aorta, using a tissue contact time of 45 min (Robertson *et al.*, 1992). However, the most striking difference between GR117289 and losartan in this preparation, is the way in which the two compounds displace the concentration-response curves to AII. Losartan caused a concentration-dependent, parallel, rightward displacement, without significantly affecting the maximum contractile response to AII. In contrast, GR117289 caused a concentration-related, insurmountable suppression of the AII concentration-response-curve. This profile of action could be explained by a number of different mechanisms: GR117289 may be (1) a 'non-specific' antagonist, (2) an allosteric antagonist, (3) an irreversible antagonist, or (4) a slowly reversible (pseudo-irreversible) antagonist, of AII.

Is GR117289 a 'non-specific' antagonist?

In this context, the term 'non-specific' is used to describe an antagonist which blocks, at some point, the chain of events leading to the production of a response by AII. Alternatively, the compound may antagonize at a receptor unrelated to angiotensin.

Even at the relatively high concentration of 1 μM, GR117289 did not affect contractile responses induced by 5-HT or phenylephrine in the rabbit aorta. This indicates not only that GR117289 has a low affinity ($pK_B < 6$) for both 5-HT_2 receptors and α_1-adrenoceptors in this tissue, but also that GR117289 does not interfere with the signal-transduction process that mediates the contraction to AII since AII (Griendling *et al.*, 1989), 5-HT (Roth *et al.*, 1986) and phenylephrine (Homcy & Graham, 1985), all induce smooth muscle contraction by receptor mediated hydrolysis of phosphatidylinositol leading to increased $[Ca^{2+}]_i$. Thus, these data suggest that the site of action of GR117289 is the AT_1 receptor macromolecule or a closely related (e.g. allosteric) site.

Further evaluation in other functional and radioligand binding studies has shown that, at $\geqslant 1$ μM, GR117289 has no detectable affinity for a wide range of receptors/binding sites/enzymes including adrenoceptors (α_2-, β_1-, β_2-), adenosine (A_1, A_2), bradykinin, dopamine (D_1, D_2), 5-hydroxytryptamine (5-HT_1, 5-HT_3), γ-aminobutyric acid ($GABA_A$, $GABA_B$), glycine, muscarinic (M_1, M_2), opioid (μ-, δ-, κ-), neuropeptide Y and neurotensin receptors, ACE, renin or adenylate cyclase (unpublished observations).

Is GR117289 an allosteric antagonist?

An allosteric antagonist is one that binds to a site on the receptor macromolecule or cell membrane, close to, but not at, the site at which the agonist binds. Nevertheless, occupation of the allosteric site by the antagonist impairs the ability of the agonist-receptor complex to generate a response. Such a phenomenon has been proposed by Kaumann & Frenken (1985) to explain the insurmountable suppression, by methysergide, of vasoconstrictor responses to 5-HT in calf coronary arteries. The functional experiments described in this report cannot exclude the possibility that GR117289 behaves as an allosteric antagonist of AII. However, in radioligand binding studies (see below), GR117289 was shown not to affect the rate of dissociation of [^{3}H]-AII from AT_1 receptors in rat liver membranes, as measured in isotope dilution experiments. Therefore, provided that GR117289 binds to AT_1 receptors in the rabbit aorta and rat liver membranes in a comparable manner, an allosteric mechanism of action in the aorta is unlikely.

Is GR117289 an irreversible antagonist?

Insurmountable antagonism can also occur when an antagonist forms an irreversible covalent bond with the receptor, such that the receptor number is effectively reduced to the point where a full agonist response cannot be achieved. However, it is unlikely that GR117289 binds irreversibly to AT_1 receptors. Evidence for this view comes from two main observations. Firstly, although the extent of the blocking action of GR117289 is progressive over 3 h of incubation, increasing the incubation time to 4 h did not appear to result in any greater degree of antagonism. Thus, GR117289 seemed eventually to reach equilibrium with AT_1 receptors in the rabbit aorta. Secondly, the degree of suppression, induced by GR117289, of the maximal response to AII, was reduced by co-incubation with losartan. Increasing concentrations of losartan displaced the concentration-response curve to AII, in the presence of GR117289, upward and to the right (Figure 4). If GR117289 had bound covalently to the AT_1 receptor, then co-incubation with a competitive antagonist would not have been expected to have any effect on the AII maximum.

Is GR117289 a slowly reversible (pseudo-irreversible) antagonist?

A further explanation of insurmountable antagonism is that slow dissociation of the antagonist from the receptor occurs. In this situation, the agonist cannot reach equilibrium with the antagonist/receptor complex under the time constraints of the experiments (see Craig *et al.*, 1990). In other words, the antagonist, once bound to the receptor, may dissociate from it so slowly as to appear irreversibly bound (pseudo-irreversible antagonism). Thus, a simple explanation for the findings of the experiments described above in which GR117289 and losartan were co-incubated, is that GR117289 dissociates slowly from the AT_1 receptors but, as it does, vacated receptors become occupied by losartan. Subsequently administered AII then competes for receptors occupied by losartan, as well as those still occupied by GR117289. As the concentration of losartan is increased, proportionally more receptors are occupied by losartan than by GR117289. Because AII can surmount the antagonism exerted by losartan, the maximal response to AII increases in relation to the proportion of receptors occupied by losartan rather than by GR117289. If GR117289 had bound irreversibly to the AT_1 receptors, co-incubation with losartan would merely have

produced further rightward displacement of the already suppressed AII curves. Similar to the present data, Wienen *et al.* (1990) and Wong & Timmermans (1991), demonstrated that the suppression of the AII curve produced by Sar^1Ile^8-AII and EXP3892, respectively, could also be reversed by losartan in rabbit aorta. More recent data (Entzeroth *et al.*, 1991) have confirmed and expanded these observations.

For this explanation of the interaction between GR117289 and losartan to be valid, it is necessary that losartan reaches equilibrium with the AT_1 receptor more rapidly than GR117289. In the present study, no experiments were conducted to determine whether losartan had reached equilibrium within the 45 min incubation period. However, other published evidence suggests that equilibrium is achieved very quickly. Using incubation times of less than 45 min, other groups have reported pA_2 values for losartan against AII in the rabbit aorta, similar to the value (8.2) obtained by Robertson *et al.* (1992) (e.g. Rhaleb *et al.*, 1991, 10 min incubation time, pA_2 8.27; Wong *et al.*, 1990b, 15 min incubation time, pA_2 8.48). It would therefore appear that, unlike GR117289, losartan reaches equilibrium with the AT_1 receptor rapidly, and certainly within 45 min. Further evidence in support of this concept is provided by the results shown in Figure 5, in which a single concentration of losartan was co-incubated for varying periods of time after pretreatment with GR117289. In these experiments, similar rightward displacements of the AII curve were seen after 2 min or 45 min incubation with losartan, again suggesting a rapid equilibration of losartan with the receptors. Figure 5 also shows that the increase in the maximum response to AII, in the presence of both antagonists, was progressive as the duration of losartan incubation was increased. This may be a measure of the slow rate of dissociation of GR117289 from the receptor.

The results obtained from the radioligand binding studies broadly support those from functional studies. In rat liver homogenates, the pK_i estimate for GR117289 was 8.7 ± 0.1. This is lower than was obtained in the rabbit aorta ($pK_B = 9.8$) and the Hill coefficient suggested that GR117289 and [^{3}H]-AII competed reversibly for the binding sites. However, it should be remembered that the competing agents were administered simultaneously in the binding studies, whereas the aorta was exposed to GR117289 for 3 h before challenge with AII. Pre-incubation of liver membranes with GR117289 for 1 h before addition of [^{3}H]-AII had little effect on the affinity estimate for GR117289. It was impractical to use longer pre-incubation times to determine whether the affinity of GR117289 would increase further.

The failure of GR117289 to increase the rate of dissociation of [^{3}H]-AII from liver membranes, following isotope dilution with excess, unlabelled AII argues against the binding of GR117289 to an allosteric site on the AT_1 receptor macromolecule. However, examination of the data shown in Figure 7 shows that the nature of the binding of GR117289 to liver membranes is characteristic of neither a simple, competitive antagonist, nor of a wholly irreversible antagonist. GR117289 both reduced the B_{max} and increased the K_d of [^{3}H]-AII binding. These findings are, therefore, consistent with the view that GR117289 may be a slowly reversible antagonist at AT_1 receptors.

Direct evidence for occupation of AT_1 receptors on rabbit aorta by GR117289, was obtained from binding studies carried out on smooth muscle membranes from this tissue. However, the low density of receptors present in these preparations meant that the experimental conditions were different from those previously used with rat liver membranes. In particular, the BSA concentration was 0.1% w/v in these experiments. This almost certainly accounts for the low pIC_{50} value (7.6 ± 0.1) obtained for GR117289 in this preparation, a view supported by the finding of a similarly low pIC_{50} (7.8 ± 0.1) in rat liver membranes used under identical conditions. Chiu *et al.* (1991) have previously reported that BSA inhibits the binding of several di-acid analogues of losartan. Thus, it is highly likely that GR117289 occupies a common binding site (i.e. receptor) in the two tissues, and that the measurements of potency obtained under these experimental conditions are underestimates of its true affinity.

The accuracy of the estimate of the dissociation constant made for GR117289, after 3 h incubation in the rabbit aorta, is debatable. It assumes that GR117289 simply occupies the same receptor site as AII and that their interaction reflects this phenomenon. However, the potency estimate made from functional studies is approximately 5 times higher than that determined from binding studies. This may be attributable to differences in experimental protocols (e.g. temperature, pretreatment time, whole tissue or membranes, the presence or absence of BSA). Although the results obtained in experiments in which rabbit aortic strips were continuously superfused with drug-free physiological salt solution for 3 h, after 45 min exposure to GR117289, are consistent with its being a very slowly dissociating antagonist, other factors may contribute to the long-lasting inhibition of responses to subsequently administered AII. In particular, GR117289 is a highly lipophilic agent (cLog P = 7.5). Thus, it is conceivable that the profile of activity of GR117289 is attributable, at least in part, to retention or, perhaps, even concentration within the membrane lipid. Apart from making it difficult to remove GR117289 by washing, this property might influence the estimation of its affinity for the angiotensin receptor in the rabbit aorta. For example, GR117289 might modulate the interaction between AII and its active site, not by changing its affinity (see above), but by reducing its efficacy as a result of altering the balance between receptor internalisation and expression, as has been suggested for some peptide antagonists of AII (Liu *et al.*, 1992). Thus, a smaller stimulus would be generated for any given agonist concentration. This would be reflected by a suppression, with little displacement, of the AII concentration-response curve. For this reason, the pK_B of 9.8 for GR117289 in this preparation should be regarded as no more than an approximation of its true affinity.

Taken as a whole, the data suggest that the most likely explanation for the insurmountable antagonism of AII by GR117289 in the rabbit aorta is attributable to its slow association with, and dissociation from, the AT_1 receptor (pseudo-irreversible antagonism). However, other explanations of the data are possible. For example, de Chaffoy de Courcelles *et al.* (1986) have described a receptor-transducer coupling model to explain insurmountable antagonism at 5-HT_2 receptor sites. In this model, an insurmountable antagonist produces its effect by binding to the receptor in such a way as to induce a conformational change in the receptor, which subsequently results in a decrease in the efficiency of stimulus-response coupling. Wong & Timmermans (1991) have tentatively applied this model to explain the insurmountable antagonism produced by EXP3892 (2'propyl-4'trifluoromethyl-5'-carboxylic acid derivative of losartan) at angiotensin AT_1 receptors in the rabbit aorta. In this model, competitive, surmountable antagonists can take the place of insurmountable antagonists, and this would explain the interaction we have observed between losartan and GR117289. However, it is not possible to distinguish between this type of interaction and pseudo-irreversible antagonism.

There are several precedents in the angiotensin literature which suggest that insurmountable antagonism is not unique to GR117289. For example, in rabbit aortic strips, suppression of the AII curve has been also reported for sarcosine substituted peptide angiotensin receptor antagonists such as Sar^1-$Cys(Me)^8$-AII (70% suppression; Freer *et al.*, 1980), Sar^1Ile^8-AII (40–90% suppression; Wienen *et al.*, 1990) or Sar^1-Ala^8-AII (saralasin, 50% suppression; Freer *et al.*, 1980; Chiu *et al.*, 1990). Similar findings have been made with non-peptide antagonists such as EXP3174 (5' carboxylic acid metabolite of losartan, 40% suppression; Wong *et al.*, 1990c)

and EXP3892, (40% suppression; Wong & Timmermans, 1991).

In conclusion, the present studies suggest that GR117289 is a potent, non-peptide, specific, selective and insurmountable, angiotensin AT_1 receptor antagonist. The mechanism of the insurmountable antagonism is debatable, but the data are consistent with GR117289 being a slowly reversible (pseudo-irreversible) antagonist.

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