ABSTRACT

Angiotensin II and endothelin-1 activate their respective AT₁ and ETA receptors on vascular smooth muscle cells, producing vasoconstriction, and both peptides are implicated in the pathogenesis of essential hypertension. Angiotensin II potentiates the production of endothelin, and conversely endothelin augments the synthesis of angiotensin II. Both AT₁ and ETA receptor antagonists lower blood pressure in hypertensive patients; thus, a combination AT₁/ETA receptor antagonist may have greater efficacy and broader utility compared with each drug alone. By rational drug design a biphenyl ETA receptor blocker was modified to acquire AT₁ receptor antagonism. These compounds (C and D) decreased Sar-Ile-Angiotensin II binding to AT₁ receptors and endothelin-1 binding to ETA receptors, and compound C inhibited angiotensin II- and endothelin-1-mediated Ca²⁺ transients. In rats compounds C and D reduced blood pressure elevations caused by intravenous infusion of angiotensin II or big endothelin-1. Compound C decreased blood pressure in Na⁺-depleted spontaneously hypertensive rats and in rats with mineralocorticoid hypertension. Compound D was more efficacious than AT₁ receptor antagonists at reducing blood pressure in spontaneously hypertensive rats, and its superiority was likely due to its partial blockade of ETA receptors. Therefore compounds C and D are novel agents for treating a broad spectrum of patients with essential hypertension and other cardiovascular diseases.

Human essential hypertension involves two powerful and well characterized vasoconstrictors angiotensin II (Ang II) and endothelin-1 (ET-1). Ang II activation of AT₁ receptors on arterial smooth muscle cells causes vasoconstriction and increases blood pressure (van den Meiracker et al., 1995), and AT₁ receptor antagonists reduce blood pressure in patients with essential hypertension (Lee and Brunner, 1993). ET-1 activation of ETA and/or ETB receptors induces vascular smooth muscle cell contraction and increases blood pressure (Yanagisawa et al., 1988), and the ETA/ETB receptor antagonist bosentan decreases diastolic blood pressure in patients with essential hypertension (Krum et al., 1998).

Emerging experimental evidence suggests an interplay between the angiotensin II and endothelin endocrine/paracrine systems. For example, Ang II increases preproendothelin mRNA expression in endothelial cells (Imai et al., 1992). Ang II stimulates the release of ET-1 from endothelial cells that involves AT₁ receptors, Ca²⁺ mobilization, and activation of protein kinase C (Emori et al., 1991). Ang II infusion into rats increases aortic ET-1 content 3-fold, and this response is blocked by the AT₁ receptor antagonists losartan (d’Uscio et al., 1998). Conversely, ET-1 treatment of pulmonary endothelial cells stimulates the conversion of Ang I to Ang II (Kawaguchi et al., 1990). BQ-123 (a peptidic ETA receptor antagonist) shifted the Ang II-mediated contraction response curves of rabbit aortas to the right (Webb et al., 1992), and ETA receptor antagonism inhibits vasoconstriction due to...
Ang II (Wenzel et al., 2001). The interaction between Ang II and ET-1 is further exemplified by the fact that low doses of either Ang II or ET-1 infused into rats had no effect on blood pressure, but simultaneous infusion of low doses of both peptides increased blood pressure dramatically (Yoshida et al., 1992).

Because Ang II and ET-1 similarly cause hypertension and copromote the other’s actions, it is plausible that concomitant blockade of both angiotensin and endothelin endocrine/paracrine pathways may lead to enhanced blood pressure reductions. The physiological benefit of dual Ang II and ET-1 blockade was demonstrated in hypertensive transgenic rats that overexpress the human renin gene (Ren-2 rats). Specifically, losartan (AT1 receptor antagonist) and SB-290670 (ETA/ETB receptor antagonist) each decreased blood pressure, whereas the combination of losartan and SB-290670 produced an additive reduction in blood pressure compared with either drug alone (Gardiner et al., 1995). A combination of losartan and a nonselective ETA/ETB receptor antagonists reduced mean arterial pressure more than monotherapy in spontaneously hypertensive rats (SHR), stroke-prone SHR, and Dahl salts-sensitive hypertensive rats (Ikeda et al., 2000). Here, we report on the discovery of novel dual action receptor antagonists (DARAs) that 1) simultaneously antagonize AT1 and ETα receptors, 2) inhibit Ang II and big-ET1-mediated hypertension, 3) are effective at lowering blood pressure in renin-dependent and renin-independent models of hypertension, and 4) are more efficacious than an AT1 receptor blocker at lowering blood pressure in SHR.

Materials and Methods

Radioligand Binding Studies

Angiotensin II. Human AT1 receptor affinity was determined using a previously described standard binding assay (Dickinson et al., 1994). Human recombinant AT1 receptor membranes (catalog no. 6110533, Biosignal; PerkinElmer, Shelton, CT) with a 0.8 pmol/mg membrane protein at a concentration of 1 mg/mL was diluted from 0.25 to 7.25 μL with buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 nM EDTA, and 0.1% bovine serum albumin). Diluted membranes (150 μL) were incubated with 10 μL of radioligand (0.2 nM 125I-Sar-Ile-Angiotensin II) and 10 μL of 0.1% DMSO vehicle in buffer, or increasing concentrations of compound or 10 μM angiotensin II. The membranes were incubated for 60 min at 37°C. Bound and free radioligand were separated and counted as described previously (Dickinson et al., 1994). 125I-Sar-Ile-Angiotensin II binding to rat aortic smooth muscle cells (RASMCs) was performed similarly to the human AT1 binding assay. RASMCs were plated in 96-well plates (Costar Corning Inc., Acton, MA) for 48 h in HEBES-buffered Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal calf serum to achieve 90% confluence. At the time of the assay, RASMCs were incubated with DMEM, 0.1% bovine serum albumin, and 0.01% sodium azide. Increasing concentrations of compound (50 μL) or 10 μM Ang II (50 μL) or 0.1% DMSO vehicle in buffer (50 μL), 0.2 nM of 125I-Sar-Ile-Angiotensin II (50 μL), and 100 μL of buffer made up the final 200-μL volume. Angiotensin II binding was performed at room temperature for 2 h on a shaker. The assay was stopped by withdrawing the assay mixture from the wells and quickly rinsing of the wells twice with 200 μL of cold PBS. Then, 200 μL of 1% Triton X-100 solution containing 0.1% BSA in distilled water was added for 15 min at room temperature to solubilize the cells. After solubilization, 150 μL of the mixture was transferred to tubes and placed in a Cobra gamma counter (PerkinElmer). IC50 values were determined by curve fitting software (SigmaPlot, SPSS Science, Inc., Chicago, IL) and inhibition constants (Ki) were calculated from the IC50 values based on the average of at least five experiments.

Endothelin-1. Human ETα affinity studies were performed as described previously (Webb et al., 1998). Stably transduced CHO-K1 cells expressing either the human endothelin A or endothelin B receptors were cultured in Ham’s F-12 media (Invitrogen) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), supplemented with 300 μg/mL Genetecin (G-418; Invitrogen) and maintained at 37°C with 5% CO2 in a humidified incubator. Twenty-four hours before assay, the cells were treated with 0.25% trypsin-EDTA and were seeded in Falcon, 96-well tissue culture plates at a density of 1.8 × 104 cells/well. For the binding assay, culture media were aspirated from each well, and the monolayers were washed with 50 μL of PBS (Mg2+/Ca2+-free). The binding assay was performed in a total volume of 125 μL consisting of assay buffer (50 mM Tris, pH 7.4, including 1% BSA and 2 μM phosphoramidon), and 25 μL of either 500 nM ET-1 (to define nonspecific binding) or competing drug. The reaction was initiated with the addition of 25 μL of 0.25 nM 125I-ET-1 (PerkinElmer). Incubation was carried out with gentle orbital shaking, at 4°C for 4 h (equilibrium binding conditions). The reaction was terminated by aspiration of the reaction buffer and two subsequent washes with cold PBS (Mg2+/Ca2+-free). The cells were disassociated by the addition of 100 μL of 0.5 N NaOH followed by incubation for 40 min. Samples were then transferred into tubes for counting in a Cobra gamma counter (PerkinElmer). Similar conditions were used for rat ETα receptor affinity studies in rat A10 cells. IC50 values were determined by curve fitting software (SigmaPlot) and Ki values were calculated from the IC50 values based on the average of at least five experiments.

Calcium Mobilization. Fetal rat aortic smooth muscle A10 cells, and RASMCs were used to monitor ETα and AT1 receptors, respectively. Cells were grown at 37°C under 5% CO2 in DMEM supplemented with 10% Hyclone fetal calf serum and 200 μg/ml G-418. Cell monolayers were trypsinized and split to 1:5 for RASMCs and 1:10 for A10 cells every 48 h to reach 85% confluence. Approximately 50,000 cells were seeded in each well of a 96-well black-sided, clear-bottomed polytetrafluoroethylene plate and incubated for 36 to 48 h before use at 85% confluence. The growth medium was aspirated and the cells were loaded with 4 μM Fluo-3 (100 μL/well; Molecular Probes, Eugene, OR) in buffer A (10 mM HEPES pH 7.4, Hanks’ salts, and 0.1% BSA) and 2.5 mM probenecid (Molecular Probes) for 1 to 3 h. The loading buffer was then replaced with 120 μL of buffer A per well.

FLIPR (Molecular Devices Corp., Sunnyvale CA) was operated under the following conditions: laser power 0.5 W, 488-nm excitation wavelength, exposure time 0.2 to 1.0 s, camera lens F stop E2, sample interval 1 to 3 s, sample addition 40 μL, dispense speed 40 μL/s, and pipetter height 20 μL below fluid level. Antagonism of ETα and ATα-mediated Ca2+ transients was determined by adding compounds to Fluo-3-loaded A10 or RASM cells for 10 min preincubation followed by addition of the agonists. Irbetasartan and compound A or C were added in 40 μL (5× concentration) to cells in 120 μL of buffered media in all 96 wells, and fluorescence was monitored for 7 min. After an additional 3 min, ET-1 or Ang II in 40 μL (5× concentration) was added by FLIPR to all 96 wells and the fluorescence was recorded every second for 7 min. The final concentrations of irbesartan were 0, 0.3, 1, and 3 nM in AT1 assay. The final concentrations of compound A were 0, 0.3, 1, 3, 10, and 30 nM in ETα assay. The final concentrations of compound C were 0, 0.3, 1, 3, 10, and 30 nM in AT1 assay and 0, 1, 3, 10, 30, and 100 nM in ETα assay. The final concentration of Ang II and ET-1 concentrations were 0.1, 0.3, 1, 3, 10, 30, 100, and 300 nM. The final concentration of DMSO was less than 0.1%. Peak heights were recorded as fluorescent values (arbitrary counts) from the charge-coupled device camera.

Agonist-mediated fluorescence responses were calculated from each well from minimum and maximum fluorescence counts, which was downloaded from FLIPR. Fluorescence responses were averaged from duplicate or triplicate on each plate. The measurements were from three plates, and standard deviations were calculated.
values were calculated from the four-parameter logistic equation Y = A + (B – A)/(1 + ((C/x)^n)) using SigmaPlot. A and B represent minimum and maximum fluorescence values, C the EC_{50} value, and D the Hill exponent. \( K_{a} \) apparent was calculated from Schild analysis (Arumullakshana and Schild, 1959) or from the equation \( K_{a} = \text{[compound concentration]} / \text{slope} - 1 \). The slope was derived from regression analysis of a plot of \( 1/A \) versus \( 1/A' \), where A and A' were equiactive agonist concentrations in the absence or presence of antagonists, respectively (Robertson et al., 1992). EC_{50} values were calculated base on the average of at least five experiments.

**Pressor Studies in Normotensive Rats.** All the in vivo studies were approved by the Bristol-Myers Squibb Animal Care Committee in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research and National Research Council, published by National Academy Press, 1996).

**Angiotensin II.** Pressor studies have been described previously (Cazoubon et al., 1993). Conscious and unrestrained Sprague-Dawley rats (8 weeks old) with indwelling carotid artery and jugular vein catheters were purchased (Hilltop, Scottsdale, PA). On arrival, they were acclimated for a minimum of 3 days to a 12-h light/dark cycle and were fed chow and water ad libitum. Eighteen hours before the experiment, the rats were fasted. On the day of the study the conscious rats were placed in boxes to allow for unrestricted movement and their arterial catheters were connected to a pressure transducer and a polygraph (Gould Instruments, Valley View, OH) for measurements of blood pressure and heart rate. An i.v. bolus injection of human Ang II (1 nmol/kg) causes a rapid and transient increase in mean blood pressure of 40 mm Hg, lasting 3 min (i.e., a pressor response), that is inhibited by AT_{1} receptor antagonists. Rats were gavaged with vehicle and immediately thereafter the first bolus i.v. injection of Ang II served as the control pressor response. Irbesartan (10 and 30 \( \mu \)mol/kg) and compounds C and D (10, 30, or 100 \( \mu \)mol/kg) were given by oral gavage, and the rats were rechallenged with Ang II at 5, 15, 30, 45 60, 90 120 150, 180, 210, and 240 min. There were six to eight rats per drug dose. The difference between the maximum Ang II mean blood pressure increase before and after drug was reported as percentage of inhibition of the Ang II pressor effect. ED_{50} values were determined by plotting the maximum percentage of inhibition of Ang II pressor response for each drug dose and using a curve fitting program (SigmaPlot).

**Big Endothelin-1.** This study was performed as described previously (Wing et al., 1995). Sprague-Dawley rats were gavaged as described above. Intravenous bolus injection of big endothelin-1 (bigET-1; 1.0 nmol/kg) into conscious normotensive rats causes a gradual and sustained increase in blood pressure of 37 mm Hg, lasting approximately 60 min, that is inhibited by ET_{A} receptor antagonists. An oral dose of vehicle followed by initial challenge of bigET-1 established a control response to the agonist. Compound A (3 and 30 \( \mu \)mol/kg) and compounds C and D (10 and 30 \( \mu \)mol/kg) were administered after control bigET-1 pressor response at 0 min, and the rats were rechallenged with bigET-1 at 15, 105, and 195 min after drug dosing. There were six to eight rats per drug dose. The gradual rise and decline of mean arterial blood pressure after i.v. bigET-1 was measured as the area under the curve using computerized image analysis (Q500MC; Leica, Bannockburn, IL), and difference between the area under the curve generated by bigET-1 before and after each dose of drug was expressed as percentage of inhibition of the bigET-1 pressor response. ED_{50} values were determined as described previously.

**Measuring Blood Pressure in Na^{+}-Depleted SHR and in Deoxycorticosterone (DOCA)-Salt Rats**

**Na^{+}-Depleted SHR.** Male SHR (13 weeks old; Taconic Farms, Germantown, NY) were used in this study. Rats were maintained on standard chow and 12-h light cycle. The SHR were fed low sodium diet (0.1 mg sodium/g chow), and furosemide (2.5 mg/kg i.m.) was administered to each rat once daily for 3 days preceding evaluation to enhance a renin-dependent state (Kitami et al., 1989; Lacour et al., 1994). One day before administering the first furosemide dose, an indwelling femoral artery catheter was implanted to measure blood pressure and heart rate. Rats were fasted overnight before the study. On the day of the study, conscious rats were placed in boxes that allowed unrestricted movement, and their catheters were connected to a pressure transducer and a polygraph (Gould Instruments). The SHR were orally gavaged once with vehicle, or irbesartan (30 \( \mu \)mol/kg) or compound C (30 or 100 \( \mu \)mol/kg), and blood pressure and heart rate were recorded at 5, 15, 30, and 45 min and at 1, 1.5, 2, 2.5, 3, 3.5, 4.5, and 5 h. There were eight to nine rats per group. After the last measurement, the catheters were sealed and the rats were returned to their cages for food and water. Twenty-four hours after dosing blood pressure and heart rate were again measured.

**DOCA-Salt Rats.** Male Sprague-Dawley rats (8 weeks old; Harlan, Indianapolis, IN) were acclimated to a 12-h light/dark cycle and fed standard chow and drank water ad libitum. Induction of mineralocorticoid hypertension was performed as described previously using DOCA treatment (Seymour et al., 1991). Rats were anesthetized with pentobarbital (65 mg/kg i.p.), and under aseptic conditions an incision in the flank was made and the renal artery and vein were ligated and the kidney removed. The wound was closed and a 100- \( \mu \)g DOCA pellet was placed s.c. between the shoulder blades on the back. After several days of recovery the rats received 0.9% NaCl and 0.2% KCl in the drinking water. Three weeks after unilateral nephrectomy an indwelling femoral artery catheter was implanted as described above. Fasted DOCA-salt rats were orally gavaged with compounds A (30 \( \mu \)mol/kg) and C (30 and 100 \( \mu \)mol/kg), and blood pressure and heart rate were recorded as described above. For the Na^{+}-depleted SHR and the DOCA-salt rat studies, mean arterial blood pressure and heart rate were averaged for the 24-h period for each rat group, and the groups were compared by an analysis of variance followed by a post hoc Dunnett’s tests.

**Measuring Blood Pressure in Telemeterized SHR.** Male SHR (15 weeks old; Taconic Farms) were prepared for telemetry measurements of blood pressure and heart rate (Data Sciences International, St. Paul, MN) as described previously (Webb et al., 1998). Catheters were inserted in the base of the abdominal aorta just above the iliac bifurcation, and the telemetry units were anchored adjacent the aorta. Seven days after surgery, the SHR were used. Three groups of SHR were orally dosed with vehicle, irbesartan, or compound D (600 \( \mu \)mol/kg doxistrong salt/rat/group). SHR received 1, 3, 10, 30, and 100 \( \mu \)mol/kg/day irbesartan or vehicle compound D for 7 days at each dose for a total of 35 days of treatment. Blood pressure and heart rate were continuously monitored for 35 days. Statistical analysis of blood pressure and heart rate differences between groups during a 24-h period were tested with a linear model using SAS PROC MIXED and Hochberger’s step-up procedure was applied to compensate for the multiple comparisons.

**Pressor Studies in SHR**

**Angiotensin II and Big Endothelin-1.** Pressor studies in SHR (15 weeks old; Taconic Farms; n = 4–6/group) were performed as described previously (Cazoubon et al., 1993). Three groups of SHR (4–7 rats/group) were dosed with vehicle, irbesartan, or compound D as in the SHR telemetry study (i.e., 1, 3, 10, 30, and 100 \( \mu \)mol/kg for 7 days at each dose). Ang II and bigET-1 pressor responses were performed on the first and last day of each drug dose in the SHR at 20, 40 min and 60, 150 min postgavage, respectively, during the 35-day treatment period. The Ang II and bigET-1 pressor responses of drug treated SHR were compared with the Ang II and big endothelin pressor responses in vehicle-treated SHR. The ED_{50} values were determined as described previously. Another five SHR were dosed with vehicle for 2 days followed by 100 \( \mu \)mol/kg/day compound E for 7 days. BigET-1 pressor test was run on day 7 of dosing compound E and compared with bigET-1 pressor effect in vehicle-treated SHR. Also baseline MAP was recorded in the same SHR before dosing.
compound E (day 1) and before bigET-1 pressor test on day 7. The percentage of inhibition of the Ang II pressor test by irbesartan and compound D and the percentage of inhibition of the bigET-1 pressor test by compounds D and E were compared with a unpaired Student’s t test with the Welch adjustment for unequal variances.

**Results**

**Rationale of DARA Drug Design**

The ETA receptor antagonist compound A (Murugesan et al., 2000) shares the same biphenyl core as a large number of AT1 receptor antagonists, including irbesartan (Fig. 1). In addition, ETA receptor antagonists and AT1 receptor antagonists share the requirement of an acidic group at the 2-position of the biphenyl moiety (a tetrazole for irbesartan and an N-isoxazolylsulfonamide for compound A). Thus, merging the structures of the ETA receptor antagonists compound A with those of the biphenyl AT1 antagonists (e.g., irbesartan) resulted in compound B, which had potent dual antagonistic activity for both the AT1 and ETA receptors (Fig. 1; Table 1). Because the N-isoxazolylsulfonamide was an acidic group required for potent ETA receptor affinity, it was fortuitous that this acidic group was tolerated by AT1 receptors (Fig. 2). The ability to significantly enhance ETA receptor affinity has been demonstrated with the introduction of certain substituents at the 2-position of the biphenyl ring, e.g., compound E (Murugesan et al., 2003). This approach can improve ETA receptor affinity over 100-fold in some cases, and this strategy was seen as a key option in the design of an AT1/ETA dual receptor antagonist (Fig. 2; Table 1). Our drug design plan was to garner as much AT1 receptor affinity as possible using a potent “AT1 receptor antagonist heterocycle” at the top of the biphenyl scaffold and then enhancing ETA receptor affinity with a potent 2-substituent (Fig. 2). This approach led to the design, synthesis, and discovery of potent DARA compounds C and D (Fig. 1; Table 1).

**Receptor Binding**

DARA compounds C and D inhibited 125I-Sar-Ile-Angiotensin II binding to rat or human AT1 receptors and 125I-ET-1 binding to rat or human ETA receptors in a concentration-dependent and monophasic manner (Fig. 3, A and B; Table 1). Compound D was 13-fold more potent than compound C at the AT1 receptor, whereas compound C was 5-fold more potent than compound D at the ETA receptor. Both compounds were inactive against human AT2 and ETB receptors (Table 1) as well as many other G protein-coupled receptors, enzymes, and ion channels (data not shown).

**Calcium Mobilization**

DARA compound C and the reference compounds irbesartan and compound A (ETA receptor antagonist) were tested for their ability to inhibit Ca2+ signaling after Ang II stimulation of RASMCs and ET-1 stimulation of rat fetal A10 cells. Ang II concentration-response curves (0–3 nM) were generated in the absence or presence of irbesartan or compound C. Irbesartan (0.3–3 nM) shifted the Ang II curve to the right and decreased the maximal response by up to 90%. Irbesartan therefore functioned as an insurmountable antagonist. Compound C exerted similar insurmountable antagonism of Ang II-mediated Ca2+ responses, although higher concentrations (10–100 nM) were necessary (Fig. 4A). Because the inhibition by both antagonists was insurmountable, a classical Schild analysis was not possible; therefore, a \( K_a \) apparent was calculated (see Materials and Methods). Thus, compound C was approximately 30-fold weaker as a functional AT1 antagonist compared with irbesartan (Table 2).

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**Fig. 1.** Structure of ETA receptor antagonists (compounds A and E), AT1 receptor antagonist (irbesartan), and DARA (compounds B, C, and D).
Concentration-response curves to ET-1 were obtained in the absence or presence of increasing concentrations of compound C (Schild plot). The reference ETA receptor antagonist compound A (0.3–30 nM) shifted the ET-1 concentration-response curve to the right and decreased the maximal response by 10 to 60%. Compound C produced no effect at 0.3 nM, but at 3 nM produced a small rightward shift and a 16% decrease in maximal response. Higher concentrations of compound C (10 and 30 nM) evoked significant rightward shifts and decreased the maximum response by up to 37% (Fig. 4B). Thus, compound C was approximately 10-fold weaker than compound A as a functional ETA receptor antagonist (Table 2), and both were insurmountable antagonists. These data from RASMC and fetal A10 cell experiments establish compound C as a functional dual action AT₁ and ETA receptor antagonist in vitro.

Testing in Hypertensive Rats

Angiotensin II Hypertension. Infusion of i.v. Ang II into normal rats resulted in a transient increase of blood pressure of 40 mm Hg, lasting approximately 3 min. In this model, the ED₅₀ for reducing hypertension indicated that irbesartan was 5- and 3-fold more potent than that of compounds C and D, respectively, when administered i.v. (Table 3). Oral dosing of compounds in the rat Ang II hypertension model indicated that compound C was 4-fold weaker than irbesartan but that compound D was 3-fold more potent than irbesartan (Table 3).

Big Endothelin-1 Hypertension. Infusion of bigET-1 i.v. into normal rats resulted in a slow and sustained increase in blood pressure of 37 mm Hg, lasting approximately 60 min. ED₅₀ calculations demonstrated that compounds C and D administered i.v. were approximately 2- to 5-fold less potent than the reference ETA receptor antagonists (compound A) when also administered i.v. (Table 3). In this model of hypertension, oral dosing indicated that compound C is 3-fold weaker, whereas compound D was equipotent compared with compound A (Table 3). Irbesartan and compound A (i.v. and p.o.) were tested in the bigET-1 and Ang II pressor tests, respectively, and both compounds were predictably inactive (Table 3). These data indicate that compounds C and D dosed either i.v. or orally inhibited both Ang II and bigET-1-induced hypertension in a dose-dependent manner for several hours, which is consistent of the action with a DARA compound.

Na⁺-Depleted SHR. DARA compound C (30 and 100 μmol/kg) and irbesartan (30 μmol/kg) were tested in this model to evaluate their antihypertensive capabilities. Compared with the vehicle group, irbesartan (30 μmol/kg p.o.) decreased mean blood pressure 5 to 12 mm Hg during the 8 h of monitoring, and at 24 h blood pressure was still 21 mm Hg less than the vehicle group (Fig. 5A). Compound C at 30 μmol/kg p.o. had little effect; however, the 100 μmol/kg p.o. dose reduced mean blood pressure by 16 to 33 mm Hg compared with vehicle-treated rats (P < 0.01). By 24 h, mean blood pressure had returned to control levels (Fig. 5A). None of the treatments caused significant changes in heart rate.

DOCA-Salt Rats. Compound C (30 and 100 μmol/kg p.o.) and compound A (30 μmol/kg p.o.) were tested in hypertensive DOCA-salt rats to evaluate their ability to lower blood pressure. Compared with vehicle-treated rats, ETA receptor antagonist compound A (30 μmol/kg) decreased mean blood pressure by 25 to 35 mm Hg during the 8 h of continuous monitoring (P < 0.01) and by 24 h mean blood pressure had returned to normal levels (Fig. 5B). Compound C at 30 μmol/kg reduced blood pressure by 10 to 35 mm Hg during the first 8 h (P < 0.01). The 100-μmol/kg dose of compound C decreased mean blood pressure by 20 to 40 mm Hg compared with vehicle-treated rats (P < 0.01). At 24 h after either dose of compound C, mean blood pressure was similar to the vehicle group. Compared with the vehicle group, compounds A and C had no effect on heart rate in this model.

DARA versus Irbesartan in SHR. Dosing rats with either vehicle, irbesartan, or compound D at 1, 3, and 10 μmol/kg for 7 days at each dose had no effect on mean arterial pressure or heart rate. However, dosing with 30 and 100 μmol/kg of either irbesartan or compound D significantly reduced blood pressure compared with vehicle and compound

**TABLE 1**

<table>
<thead>
<tr>
<th>Kᵢ nM</th>
<th>Human AT₁</th>
<th>Rat AT₁</th>
<th>Human ETₐ</th>
<th>Rat ETₐ</th>
<th>Human AT₂</th>
<th>Human ET₂</th>
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<tr>
<td>Compound A</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.4</td>
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<td>Irbesartan</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.2</td>
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<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>Compound B</td>
<td>8.6 ± 1.0</td>
<td>3.6 ± 1.0</td>
<td>40 ± 8</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Compound C</td>
<td>10.4 ± 1.0</td>
<td>7.9 ± 1.0</td>
<td>1.9 ± 0.4</td>
<td>2.5 ± 0.8</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Compound D</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>9.3 ± 1.1</td>
<td>16 ± 4</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Compound E</td>
<td>&gt;1000</td>
<td>n.d.</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>n.d.</td>
<td>810 ± 400</td>
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</table>

n.d., not determined.

**Fig. 2.** Summary of the structure activity relationships of DARA compounds.
D was more efficacious at lowering blood pressure compared with irbesartan (Fig. 6). Statistical analyses of the first and last 24-h periods of the 10-μmol/kg doses (i.e., days 15 and 21) indicated that there were no significant difference in MAP between the vehicle, irbesartan, and compound D. For the first day of the 30-μmol/kg dose (day 22), both irbesartan and compound D reduced MAP compared with controls, whereas compound D reduced MAP greater than irbesartan (Fig. 6; Table 4). At 100 μmol/kg (days 29 and 35), this trend continued as irbesartan reduced MAP compared with controls and compound D decreased MAP compared with irbesartan (Figs. 6 and 7A; Table 4). In fact, 100 μmol/kg compound D normalized blood pressure for about 4 h from approximately 170 to 100 mm Hg in the SHR. Heart rate was also monitored in the same SHR. Compared with vehicle, 100 μmol/kg compound D elevated heart rate compared with irbesartan (Fig. 7B; P < 0.0443).

**Estimating AT₁ and ETA Receptor Blockade by Irbesartan and Compound D in SHR.** To determine whether the superior blood pressure reductions of compound D over irbesartan were due to either more potent AT₁ receptor antagonism and/or its blockade of ETA receptors, Ang II and bigET-1 pressor experiments were conducted in SHR. The pressor tests estimated the degree of in vivo AT₁ and ETA receptor blockade after treatment with either irbesartan or compound D. SHR were dosed as in the previous telemetry

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**TABLE 2**

<table>
<thead>
<tr>
<th>Functional potencies (Kᵦ, apparent) of irbesartan, compound A, and DARA compound C</th>
<th>Irbesartan</th>
<th>Compound A</th>
<th>Compound C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat AT₁</strong> Kᵦ apparent (nM)</td>
<td>0.29 ± 0.08</td>
<td>N.D.</td>
<td>9.6 ± 3.4</td>
</tr>
<tr>
<td><strong>Human ETA</strong> Kᵦ apparent (nM)</td>
<td>N.D.</td>
<td>0.41 ± 0.06</td>
<td>3.8 ± 1.6</td>
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</table>

N.D., not determined.
Ang II blockade with compound D at 100 μmol/kg was less than irbesartan (Table 5). However, the degree of DOCA-salt rats.

Ang II or bigET-1-induced hypertension in normotensive Sprague-Dawley rats. Some data was previously reported in Murugesan et al. (2002).

<table>
<thead>
<tr>
<th>Compound, i.v., p.o.</th>
<th>Inhibition of Ang II-Induced HT</th>
<th>Inhibition of bigET-1-Induced HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound A, i.v., p.o.</td>
<td>Inactive</td>
<td>0.9, 8.1</td>
</tr>
<tr>
<td>Irbesartan, i.v., p.o.</td>
<td>0.3, 10.3</td>
<td>Inactive</td>
</tr>
<tr>
<td>Compound C, i.v., p.o.</td>
<td>1.4, 39.0</td>
<td>2.1, 26.0</td>
</tr>
<tr>
<td>Compound D, i.v., p.o.</td>
<td>0.8, 3.6</td>
<td>7.6, 10.9</td>
</tr>
<tr>
<td>Compound E, p.o.</td>
<td>n.d.</td>
<td>1.0</td>
</tr>
</tbody>
</table>

ED50 μmol/kg

HT, hypertension; n.d., not determined.

Discussion

This report highlights the unique pharmacology of novel dual action AT1 and ETₐ receptor antagonists. By recognizing the structural similarities between irbesartan (an AT₁ receptor antagonist) and biphenyl ET₁ receptor antagonists (compounds A and E), we were able to merge their unique features and afford dual action receptor antagonists. Specifically, by replacing the C4'-oxazole in compound A with the imidazolinone of irbesartan, we were able to add potent AT₁ receptor antagonism and retain moderate ETₐ receptor affinity into a series of compounds that were previously selective ETₐ receptor antagonists. In essence, a potent ETₐ receptor antagonist (compound A) was converted into a potent AT₁ receptor antagonist (compound B) that still retained moderate activity at ETₐ receptors. Previous studies of biphenyl ETₐ receptor antagonists indicated that substituents at the 2'-position of the biphenyl scaffold could significantly improve ETₐ receptor affinity (Murugesan et al., 2003). Using this strategy, the ETₐ receptor potency of compound B was improved as demonstrated by compounds C and D.

In vitro binding studies demonstrated that compounds C and D antagonized rat and human AT₁ and ETₐ receptors, whereas they were inactive at human AT₂ or ETB receptors. Calcium mobilization studies demonstrated that irbesartan and compound C were insurmountable AT₁ receptor antagonists and that compounds A and C were insurmountable ETₐ receptor antagonists. A completely different series of DARA compounds were previously reported to antagonize AT₁, AT₂, ETₐ, and ETB receptors in vitro ligand binding studies, but no further in vitro or in vivo pharmacology was reported (Walsh et al., 1995). A preliminary report on compound C was published recently (Murugesan et al., 2002).

Further evidence for the dual antagonistic action of compounds C and D at the AT₁ and ETₐ receptors was demonstrated by their ability to reduce hypertension in rats caused by bolus i.v. infusions of either Ang II or bigET-1. On the other hand, compound A was ineffective at decreasing Ang II-induced hypertension, whereas irbesartan was ineffective during bigET-1-induced hypertension. SHR fed a low sodium diet and treated with a diuretic have an activated renin-angiotensin system (Kitami et al., 1989). They demonstrate reproducible reductions in blood pressure when treated with angiotensin-converting enzyme inhibitors (DiNicolantonio and Doyle, 1985) or AT₁ receptor antagonists (Lee et al., 1994), and thus are regarded as model of renin-dependent hypertension. Consistent with the actions of AT₁ receptor

Fig. 5. A, effect of irbesartan and DARA compound C on blood pressure in Na⁺-depleted SHR. B, effect of compound A (selective ETₐ receptor antagonist) and DARA compound C on blood pressure in hypertensive DOCA-salt rats.

SHR treated with compound D, there was a dose-dependent inhibition of bigET-1 induced hypertension with a maximum of 60% at 100 μmol/kg (day 35; Fig. 8B). Irbesartan did not decrease bigET-1 hypertension. Compound E was a potent and selective ETₐ receptor antagonists (Table 1) and 7 days of dosing at 100 μmol/kg produced an 80% inhibition of bigET-1 hypertension in SHR, which was similar to the 60% inhibition with compound D (P = 0.09). Dosing SHR for 7 days with compound E (100 μmol/kg/day p.o.) reduced baseline mean arterial pressure by 11% compared with the pre-drug mean arterial pressure (from 175 ± 5 to 155 ± 5 mm Hg; P < 0.01), indicating that high doses of selective ETₐ receptor antagonists alone can reduce blood pressure in SHR.
antagonists compound C and irbesartan delivered orally decreased blood pressure in this model.

Sprague-Dawley rats treated with DOCA and drinking 0.9% NaCl (DOCA-salt rats) develop hypertension that is associated with a suppression of the renin-angiotensin system and increased tissue levels of ET-1 (Fujita et al., 1995; Schiffrin et al., 1996). Consequently, ETA receptor antagonists (Bird et al., 1995; Schiffrin et al., 1997) but not AT1 receptor antagonists (Wada et al., 1995; Pollock et al., 2000) have shown antihypertensive activity in this model. Compounds A and C reduced blood pressure in hypertensive DOCA-salt rats, consistent with the actions of an ETA receptor antagonist. Therefore, DARA compound C produced dose-dependent and sustained blood pressure reductions in renin-independent and renin-independent models of hypertension. These studies indicate that compound C has potential broad utility as an antihypertensive agent.

β-Blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors and AT1 receptor antagonists are efficacious in SHR, suggesting it is a model human essential hypertension. Therefore, increasing doses of DARA compound D and irbesartan was compared in telemeterized SHR. At 30 and 100 μmol/kg/day, irbesartan reduced blood pressure compared with vehicle controls, whereas compound D significantly decreased blood pressure more than irbesartan at the 30- and 100-μmol/kg doses. To determine the degree of Ang II and ET-1 blockade with each drug in SHR, Ang II, and bigET-1 pressor studies were conducted in a separate group of rats using the same dosing regimen as in the telemetry study. At doses of 10 to 30 μmol/kg, compound D compared with irbesartan was a more potent AT1 receptor antagonists in vivo as indicated by the Ang II pressor ED50 values and

![Fig. 6. Effect of vehicle, irbesartan, and compound D on SHR mean arterial pressure at 10, 30, and 100 μmol/kg/day. Vertical lines indicate time of daily dosing at 8:00 AM.](image)

![Fig. 7. A, effect of vehicle, irbesartan, and compound D on SHR mean arterial pressure during the 7th day of dosing at 100 μmol/kg (day 35). Arrow indicates time of oral dosing. Irbesartan reduced blood pressure compared to vehicle, and compound D decreased blood pressure compared with vehicle and irbesartan. B, effect of vehicle, irbesartan, and compound D on SHR heart rate during the 7th day of dosing at 100 μmol/kg (day 35). Arrow indicates time of oral dosing. Compound D increased heart rate compared to vehicle and irbesartan (P < 0.0443). Irbesartan had no significant effect on heart rate.](image)

<table>
<thead>
<tr>
<th>Dose (μmol/kg)</th>
<th>Day</th>
<th>Comparison</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>22</td>
<td>Vehicle vs. irbesartan</td>
<td>0.0082</td>
</tr>
<tr>
<td>30</td>
<td>22</td>
<td>Vehicle vs. compound D</td>
<td>0.0001</td>
</tr>
<tr>
<td>30</td>
<td>22</td>
<td>Irbesartan vs. compound D</td>
<td>0.2847</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>Vehicle vs. irbesartan</td>
<td>0.0002</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>Vehicle vs. compound D</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>Irbesartan vs. compound D</td>
<td>0.0209</td>
</tr>
<tr>
<td>100</td>
<td>29</td>
<td>Vehicle vs. irbesartan</td>
<td>0.0001</td>
</tr>
<tr>
<td>100</td>
<td>29</td>
<td>Vehicle vs. compound D</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>29</td>
<td>Irbesartan vs. compound D</td>
<td>0.0209</td>
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<tr>
<td>100</td>
<td>35</td>
<td>Vehicle vs. irbesartan</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>35</td>
<td>Vehicle vs. compound D</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>35</td>
<td>Irbesartan vs. compound D</td>
<td>0.0010</td>
</tr>
</tbody>
</table>
could easily explain the greater blood pressure efficacy in SHR of compound D versus irbesartan at these doses. However, on the 1st and 7th day of dosing irbesartan and compound D at 100 μmol/kg (days 29 and 35), the inhibition of the Ang II pressor response was not significantly different in the SHR, suggesting a similar degree in AT₁ receptor blockade in vivo. Nevertheless, compound D reduced blood pressure to a greater extent in SHR compared with irbesartan. The reduction of blood pressure by irbesartan and compound D could have also been a result of evoking the bradykinin and nitric oxide pathways (Horning et al., 2003).

BigET-1 pressor studies in the same SHR indicated that irbesartan did not affect bigET-1-induced hypertension at any dose (data not shown), but as expected compound D decreased bigET-1 hypertension in a dose-dependent manner up to 60%, indicating incomplete ET₂ receptor antagonism in vivo. Compound E (a potent and selective ET₂ receptor antagonist) at doses of 100 μmol/kg in SHR decreased the pressor response to bigET-1 infusion by 80% and also reduced baseline hypertension after 7 days of treatment in this model. This suggests that selective and partial ET₂ receptor blockade produces lower blood pressure in SHR. Together, the superiority of compound D over irbesartan at the 100-μmol/kg doses was likely due to partial antagonism of ET₂ receptors because 1) the degree of inhibition of Ang II was similar between both compounds, 2) ET₂ receptor antagonism by compound D was demonstrable at this dose, and 3) selective ET₂ receptor antagonists decreases blood pressure in SHR.

In summary, DARA compounds represent a new class of antihypertensive agent that simultaneously antagonize AT₁ and ET₂ receptors. These compounds are effective in various forms of experimental hypertension and are more efficacious than a selective AT₁ receptor antagonist. Therefore, compounds C and D provide a novel method of treatment for essential hypertension and other cardiovascular diseases.

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References


