Pharmacological Characterization of Aprocitentan, a Dual Endothelin Receptor Antagonist, Alone and in Combination with Blockers of the Renin Angiotensin System, in Two Models of Experimental Hypertension

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ABSTRACT

The endothelin (ET) system has emerged as a novel target for hypertension treatment where a medical need persists despite availability of several pharmacological classes, including renin angiotensin system (RAS) blockers. ET receptor antagonism has demonstrated efficacy in preclinical models of hypertension, especially under low-renin conditions and in hypertensive patients. We investigated the pharmacology of aprocitentan (N-[5-(4-bromophenyl)-6-[2-[(5-bromo-2-pyrimidinyl)oxy]ethoxy]-4-pyrimidinyl]-sulfamide), a potent dual ETA/ETB receptor antagonist, on blood pressure (BP) in two models of experimental hypertension: deoxycorticosterone acetate (DOCA)-salt rats (low-renin model) and spontaneously hypertensive rats [SHR, normal renin model]. We also compared the effect of its combination with RAS blockers (valsartan and enalapril) with these RAS blockers. In hypertensive rats under sodium restriction and enalapril, addition of aprocitentan further decreased BP without causing renal impairment, in contrast to spironolactone. In hypertensive rats under sodium restriction and enalapril, addition of aprocitentan further decreased BP (statistically significant) and renal vascular resistance, and reduced left ventricle hypertrophy (nonsignificant). Aprocitentan was synergistic with valsartan and enalapril in decreasing BP in DOCA-salt rats and SHRs while spironolactone demonstrated additive effects with these RAS blockers. In hypertensive rats under sodium restriction and enalapril, addition of aprocitentan further decreased BP without causing renal impairment, in contrast to spironolactone. In conclusion, ETA/ETB receptor antagonism represents a promising therapeutic approach to hypertension, especially with low-renin characteristics, and could be used in combination with RAS blockers, without increasing the risk of renal impairment.

Introduction

Endothelin (ET)-1 is a potent vasoconstrictor peptide that also causes neurohormonal and sympathetic activation, increased aldosterone synthesis and secretion, vascular hypertrophy and remodeling, fibrosis, and endothelial dysfunction (Iglarz and Clozel, 2010). ET-1, and probably ET-2 and ET-3, can contribute to the pathogenesis of hypertension and related end-organ damage via their receptors, ETA and ETB. The ET system is involved in the regulation of blood pressure (BP) in response to salt and volume expansion, as confirmed by upregulation of this system in salt-dependent animal models of hypertension (Schiffrin, 1998b). Accordingly, dual ETA/ETB endothelin receptor antagonists (ERAs) demonstrate greater efficacy in salt-dependent/low-renin animal models of hypertension than in high/normal renin animal models (Schiffrin, 1998a).

Aprocitentan (N-[5-(4-bromophenyl)-6-[2-[(5-bromo-2-pyrimidinyl)oxy]ethoxy]-4-pyrimidinyl]-sulfamide) (Fig. 1A) is the active metabolite of macitentan, also a dual ETA/ETB ERA, which demonstrated long-term efficacy in pulmonary hypertension. Aprocitentan is a potent, orally active, dual ETA/ETB ERA with an ETA/ETB inhibitory potency ratio of 1:16, as determined by in vitro functional assays (Iglarz et al., 2008), and a long half-life (44 hours) in humans (Sidharta et al., 2018).

ET-1 production is activated by salt. The upregulation of ET-1 in hypertensive models is greater in salt-sensitive animals, in which ET receptor blockade protects against end-organ damage, than in salt-resistant animals (Schiffrin, 1998b). The ET system is upregulated in hypertensive African
Americans who have a high prevalence of salt-sensitive hypertension, but also in various conditions such as obstructive sleep apnea, diabetes, obesity, and chronic kidney disease (Belaidi et al., 2009; Iglarz and Clozel, 2010). Preclinical models mimicking these comorbidities, such as intermittent hypoxia, streptozotocin-induced diabetes, db/db diabetic mice, and remnant kidney model, are also associated with upregulation of the ET system, and blockade of ET receptors provides end-organ protection in these models (Benigni et al., 1996; Iglarz et al., 2008; Belaidi et al., 2009; Sen et al., 2012).

Because of the involvement of ET-1 not only in volume-dependent hypertension but also in neurohormonal activation, end-organ damage, and comorbidities associated with hypertension, dual ETA/ETB ERAs represent a potential new pharmacological approach in the treatment of human essential and resistant hypertension. Resistant hypertension is defined as uncontrolled BP despite triple therapy including renin angiotensin system (RAS) blockers at appropriate doses, good compliance with drug administration, and absence of secondary hypertension, and is frequently associated with intravascular volume increase and aldosterone excess (Judd and Calhoun, 2014). RAS blockade and the associated sodium depletion is the basis of the most frequently prescribed hypertension treatments, at all steps. A new antihypertensive drug presenting a novel mechanism of action would be expected to further lower BP when administered with existing treatments in patients with uncontrolled hypertension, and should therefore demonstrate additional clinical benefits (Lewington et al., 2002; Food and Drug Administration, 2018). Since the dual ETA/ETB antagonist aprocitentan targets a different pathway, it should be evaluated in combination with existing therapies, in particular RAS blockers. The combination of aprocitentan and a RAS blocker may show greater efficacy and improved safety compared with the combination of a mineralocorticoid receptor antagonist (MRA), such as spironolactone, and a RAS blocker. The combination with aprocitentan could improve safety by minimizing the risk of renal impairment and hyperkalemia, which is observed during the excessive pharmacological blockade of the renin angiotensin aldosterone axis (Abbas et al., 2015).

Therefore, we studied the pharmacology of aprocitentan alone and in combination with RAS blockers, and its dose-response relationship, in two different rat models of experimental hypertension with different levels of renin activation. We also assessed the renal safety profile of aprocitentan during sodium depletion and in combination with a RAS blocker. Overall, our data demonstrate that ET receptor blockade could be as efficacious as and safer than an MRA in hypertensive patients concurrently treated with RAS inhibitors.

Materials and Methods

Animals and Drugs. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD) and were approved by the local Basel-Landschaft cantonal veterinary office (Switzerland). All rats were maintained under identical conditions and had free access to drinking water and food. Rats were paired and housed in climate-controlled conditions with a 12-hour light/dark cycle. Wistar rats were obtained from...
Harlan Laboratories (Horst, The Netherlands) or Charles River Laboratories (Sulzfeld, Germany); spontaneously hypertensive rats (SHRs) were obtained from Harlan Laboratories. Compounds were synthesized at Idorsia Pharmaceuticals Ltd. (Allschwil, Switzerland), and were administered by gavage in volumes of 5 ml/kg body weight in gelatin 4%.

Plasma Renin Activity and ET-1 Concentrations in Rats. Sublingual blood samples were collected into EDTA-coated tubes from rats under 2.5% isoflurane anesthesia. Blood was sampled from 6- to 7-month-old male Wistar rats (n = 8), SHRs (n = 9), and deoxy-corticosterone acetate (DOCA)-salt rats (n = 9) for plasma renin activity quantification. Blood from 3-month-old male Wistar rats was sampled before and 3 hours after aproticentan (0.3–300 mg/kg, n = 4) or vehicle (n = 10) administration for ET-1 plasma concentration quantification. Plasma renin activity was measured by enzyme-linked immunosorbent assay (IBL International, Hamburg, Germany) and ET-1 concentration was measured by chemiluminescent immunosassay (Quintiglo; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany).

Induction of Hypertension, Transmitter Implantation, and Data Collection. After an acclimatization period of at least 7 days, 10-week-old male Wistar rats were anesthetized with 2.5% isoflurane (Attane; Minrad Inc., Buffalo, NY), the left kidneys were removed by flank incisions, and two pellets of 25 mg DOCA were subcutaneously implanted. The drinking water was 1% saline solution (for the DOCA-salt rats). After isoflurane-induced narcosis, DOCA-salt rats and SHRs were microsurgically implanted with a telemetry pressure transmitter (Data Sciences International, New Brighton, MN) in the peritoneal cavity and a pressure catheter was inserted into the aorta below the renal artery pointing upstream. The transmitter was sutured to the inside of the abdominal wall 1 cm from the edge of the incision, and both the abdominal musculature and the skin were closed. Buprenorphine (Tengepis, 0.05 mg/kg; Essex Chemie AG, Lucerne, Switzerland) was given subcutaneously 30 minutes before surgery and once a day for 2 days following surgery. Implanted rats were given a recovery period of at least 2 weeks before the start of treatment. BP was collected continuously using the Dataquest ART Gold acquisition system (version 3.01; Data Sciences International). Ten-second measurements of systolic, mean, and diastolic arterial pressures and heart rate (HR) were collected at 5-minute intervals, resulting in a series of 285 data points per day for each animal. Mean arterial pressure (MAP) was expressed in millimeters of mercury and HR in beats per minute. Measurements were collected for 24 hours before and up to 120 hours after single oral administration of compounds.

Acute Effects of Aprocitentan, Enalapril, Valsartan, Amloidipine, Spironolactone, and Their Combination on BP in Conscious SHRs and DOCA-Salt Rats. Single oral administration of vehicle (gelatin, 4%), or drugs was performed by gavage of a suspension or solution in 4% gelatin (n = 5–13/group). We used both maximal BP reduction and calculation of the area between the curves (ABC) to determine minimally and maximally effective doses in dose-response curves. The ABC and MAP decreases were calculated for each rat using the hourly means of BP between data collected 24 hours before administration of the compound and the data collected during the treatment period. The minimally and maximally effective doses were obtained by calculating the mean of six consecutive hours of measurement. Each telemetry experiment had its own vehicle-treated control group. For acute single dose combination studies, doses that induced partial BP decreases of 10–20 mm Hg were chosen. Doses were 100 and 10 mg/kg aproticentan, 3 and 10 mg/kg enalapril, and 10 and 30 mg/kg valsartan in SHRs and DOCA-salt rats, respectively. Doses of 1 and 10 mg/kg were selected for amloidipine and aproticentan in DOCA-salt rats, respectively (Supplemental Fig. 3). The selected dose of spironolactone was 300 mg/kg since it was the only dose inducing a significant BP decrease in both models (Supplemental Fig. 1). Of note, the BP reduction profile of spironolactone was different in DOCA-salt rats and SHRs, reflecting the difference in the mode of action of the MRA in the two models (Zannad, 1991). In DOCA-salt rats, single dose administration of spironolactone led to a rapid decrease (within 3 hours) in BP, suggesting direct inhibition of the vasoconstrictor effect of DOCA on the mineral receptors (Supplemental Fig. 1A). In SHRs, the BP reduction occurred 48 hours after single oral administration, suggesting a renal (diuretic) effect of the compound (Supplemental Fig. 1B). Since the maximal BP reduction induced by spironolactone was observed 2 days after oral administration in SHRs, valsartan or enalapril were administered 2 days after spironolactone administration to match the time point at which both drugs exerted their maximal hemodynamic effects. For combination studies in DOCA-salt rats, spironolactone was administered concomitantly with either valsartan or enalapril. The predicted additive effect was calculated by adding the BP decreases induced by both drugs compared with baseline at each time point.

Chronic Effects of Aprocitentan on BP, Renal Hemodynamics, and Left Ventricular Hypertrophy in Conscious DOCA-Salt Rats. Aprocitentan (1, 10, and 100 mg/kg per day) or vehicle was administered by oral gavage to DOCA-salt rats for 28 days (n = 16 per group). Fourteen age-matched Wistar rats administered vehicle were used as controls. In each group, six rats were implanted with a telemetry device for continuous MAP and HR recording, and 8–10 rats were used to assess renal vascular resistance, relative left ventricular weight, and plasma N-terminal pro b-type natriuretic peptide concentration. To provide baseline data, a group of six DOCA-salt rats were sacrificed 2 weeks after DOCA-salt treatment, just before initiation of aproticentan or vehicle administration. One day before the end of the treatment phase, blood from nonimplanted rats was collected through sublingual vena puncture under isoflurane (2.5%)-induced narcosis for the measurement of plasma N-terminal pro b-type natriuretic peptide concentrations (Meso Scale Discovery, Rockville, MD). At the end of the treatment phase, rats were anesthetized with 100 mg/kg inactin, placed on a thermostatically controlled heating table, and cannulated for 1) tracheotomy, 2) p-aminohippurate infusion in the right femoral vein, 3) blood sampling and hemodynamic measurements in the right femoral artery, and 4) urine collection in the bladder. Urine and plasma p-aminohippurate concentrations were measured to assess renal vascular resistance as previously described (Ding et al., 2003). Rats were then sacrificed, and hearts were removed and weighed. The left ventricle plus septum and right ventricle were separated and weighed. The ratio of the left ventricle plus septum weight to heart weight was calculated and used as an index of left ventricular hypertrophy. Left ventricles were then placed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm in thickness were stained with hematoxylin and eosin or Masson’s trichrome and examined by light microscopy for morphologic assessment as previously described (Jokinen et al., 2011).

Effect of Aprocitentan or Spironolactone in Combination with Enalapril on Renal Function in Conscious SHRs on a Low-Salt Diet. We selected the model developed by Richer-Giudicelli et al. (2004) to study the effect of the combination therapies on renal function. SHRs on a low-salt diet are sensitive to excessive blockade of the renin angiotensin aldosterone system, which has been proposed to be responsible for increased risk of renal failure in clinical trials (Azizi and Ménard, 2004; Richer-Giudicelli et al., 2004). After an acclimatization period of 1 week to a low-salt diet (0.06% salt, Granovit ref.2036, Kaiseraugst, Switzerland), SHRs implanted with the telemetry pressure transmitter were orally treated with either vehicle (gelatin 4%, n = 4) or enalapril 10 mg/kg per day (n = 19) for 6 days. Then, all enalapril-treated SHRs were randomized into two experimental groups matched for similar MAP and plasma creatinine levels. These rats received either enalapril 10 mg/kg per day plus aproticentan 10 mg/kg per day (n = 9) or enalapril 10 mg/kg per day plus spironolactone 300 mg/kg per day (n = 10) for five additional days. The MAP of all rats was recorded for 1 hour at baseline and 4 hours after the last administration of enalapril (at day 6) and combinations (at day 11). Sublingual blood samples were collected under 2.5% isoflurane anesthesia into lithium/heparin-coated tubes.
for plasma creatinine, urea, and electrolyte level quantification (AU480 chemistry system; Beckman Coulter).

**Plasma Drug Concentrations.** Plasma drug concentrations were measured in Wistar rats for the dose-response of aprocitentan and SHRs for the combination studies after oral administration alone or in combination of the drugs (n = 6 per group). Plasma samples were collected from rats under 2.5% isoflurane anesthesia 1, 2, 3, 4, 6, 8, and 24 hours after oral administration of compounds. Plasma compound quantification was performed by liquid chromatography–tandem mass spectrometry (API4000; AB SCIEX, Concord, ON, Canada).

**Expression of Results and Statistical Analysis.** Results are expressed as mean ± S.E.M. Statistical comparisons between multiple groups were performed using one-way analysis of variance, followed by a Newman–Keuls multiple comparisons post hoc test. The combination experiments for BP were conducted as factorial designs including: vehicle, compound A (aprocitentan or spironolactone), compound B (valsartan or enalapril), and the combination of compounds A and B. The mean change in MAP (measured by the ABC until the end of each compound's effect) was calculated for each group and the synergy (interaction) between two compounds was estimated as follows: mean (AB) − mean (A) − mean (B) + mean (vehicle). The interaction estimate was divided by its S.E., and then tested using a t test. A two-sided value of P < 0.05 was considered statistically significant.

**Results**

**Pharmacokinetics of Aprocitentan and Dose-Response Curve of Single Doses on Plasma ET-1 Concentrations in Conscious Normotensive Rats.** Single oral administration of 0.3–100 mg/kg aprocitentan led to dose-dependent long-lasting increases in plasma drug concentrations (Fig. 1B). We measured ET-1 plasma concentration as a marker of in vivo ETB receptor blockade, since binding of an ERA to ETB receptors increases ET-1 plasma concentration (Loßl et al., 1995). Aprocitentan dose-dependently increased the ET-1 plasma concentration in normotensive rats, with a significant effect in rats receiving doses ≥10 mg/kg versus those receiving vehicle (P < 0.01, Fig. 1C).

**Dose-Response Curve of Single Doses of Aprocitentan and Valsartan on BP in Conscious DOCA-Salt Rats.** The pharmacological activity of aprocitentan was characterized in two models of hypertension with different levels of plasma renin activity. Plasma renin activity was significantly blunted in DOCA-salt rats compared with SHRs and Wistar rats (Fig. 2A). The dose-response effects of aprocitentan and the angiotensin II type-1 receptor blocker (ARB) valsartan on maximal MAP decrease and ABC were compared in these models. Aprocitentan induced a dose-dependent decrease in BP in both models, with a greater efficacy and potency in DOCA-salt rats than in SHRs (Fig. 2B). At a dose of 100 mg/kg aprocitentan, the maximal decreases in MAP from baseline were 18 ± 4 and 29 ± 4 mm Hg and in ABC were 1046 ± 149 and 1459 ± 335 mm Hg per hour in SHRs and DOCA-salt rats, respectively. The maximal effective dose (estimated by combining the maximal BP decrease with the ABC) was 100 mg/kg, with the ED50 > 3 mg/kg in SHRs, and the maximal effective dose was 10 mg/kg, with the ED50 = 3 mg/kg in DOCA-salt rats. By contrast, valsartan induced a more pronounced BP decrease in SHRs than in DOCA-salt rats (Fig. 2C). At a dose of 10 mg/kg valsartan, the maximal decrease in MAP from baseline was 17 ± 2 mm Hg, with an ABC of 338 ± 53 mm Hg per hour in SHRs. In DOCA-salt rats, a fall in MAP, which was not statistically significant compared with vehicle, was detected at 30 and 100 mg/kg. Overall, based on the full dose-response curve profiles on BP, aprocitentan showed greater pharmacological activity in DOCA-salt rats than in SHRs and greater pharmacological activity than valsartan in both hypertensive models. When analyzing the BP-lowering profile in DOCA-salt rats, aprocitentan dose-dependently decreased MAP in a sustained manner (Fig. 3A) without affecting HR (Fig. 3B). The duration of action was prolonged (>24 hours) at doses ≥10 mg/kg, consistent with its long-lasting plasma concentrations (Fig. 1B) and half-life of 8.4 hours in rats, as previously reported (Iglarz et al., 2008).

**Dose-Response Curve of Chronic Treatment of Aprocitentan on BP, Renal Hemodynamics, and Left Ventricle in DOCA-Salt Rats.** Because of the greater involvement of the ET system in DOCA-salt rats than in SHRs, we selected the DOCA-salt model to study the efficacy of a daily treatment with aprocitentan for 28 days on MAP, HR, renal hemodynamics, and left ventricular remodeling. All untreated DOCA-salt rats developed hypertension, increased renal vascular resistance, left ventricular hypertrophy, and moderate cardiomyopathy characterized by slight interstitial fibrosis and more pronounced inflammatory cell infiltration (mainly lymphocytes) than Wistar control rats. The 10 and 100 mg/kg per day doses of aprocitentan equally decreased MAP (by 19 and 22 mm Hg, respectively) and renal vascular resistance without affecting heart rate (Fig. 4, A–C). Aprocitentan also reduced the incidence of cardiomyopathy in DOCA-salt rats (Supplemental Table 1). At doses of 10 and 100 mg/kg per day the decreases in relative left ventricular weight and N-terminal pro b-type natriuretic peptide plasma concentrations compared with vehicle-treated DOCA-salt rats were not statistically significant (Fig. 4, D and E).

**Combination of Single Doses of Aprocitentan or Spironolactone with a RAS Blocker on BP in Conscious SHRs and DOCA-Salt Rats.** In SHRs and DOCA-salt rats, coadministration of a single dose of aprocitentan and valsartan decreased MAP beyond the predicted (calculated) additive values, demonstrating synergy between the blockers of each pressor system (Fig. 5, A and B; Table 1). The combination of aprocitentan with enalapril was synergistic in DOCA-salt rats and was additive in SHRs (Fig. 5, C and D; Table 1). Conversely, a combination of either valsartan or enalapril with spironolactone was only additive and did not lead to a synergistic reduction of the MAP (Fig. 6; Table 1). No change in drug concentration was observed in animals treated with this combination when compared with monotherapy (Supplemental Table 2). The ability of aprocitentan to work on top of other background therapies in low-renin conditions was demonstrated in DOCA-salt rats, where aprocitentan further decreased BP when administered with the calcium channel blocker amlodipine (Fig. 7; Supplemental Fig. 3).

**Effect of the Combination of Chronic Treatment of Aprocitentan or Spironolactone with a RAS Blocker on BP and Renal Function in SHRs on a Low-Salt Diet.** SHRs were equipped with telemetry to match the BP lowering effects obtained during therapies, thereby avoiding any confounding hemodynamic effect on renal function. Under low-salt diet conditions, a 6-day administration of enalapril 10 mg/kg decreased BP from 154 ± 2 to 118 ± 3 mm Hg without increasing plasma urea and creatinine concentrations. Addition
Fig. 2. (A) Plasma renin activity in Wistar rats, SHRs, and DOCA-salt rats, n = 8 to 9/group; data are presented as mean ± S.E.M. Dose-response relationship on maximal mean arterial blood pressure decrease after single oral administration of aprocitentan (0.3–300 mg/kg, n = 4–11/group) (B) and valsartan (0.3–100 mg/kg, n = 6–24/group) (C) in conscious SHRs (left) and DOCA-salt rats (right) equipped with telemetry. Data are presented as mean ± S.E.M. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 compared with vehicle-treated rats.
of either spironolactone or aprocitentan to enalapril for another 5 days further decreased BP to similar levels in both groups (94 ± 6 and 92 ± 3 mm Hg, respectively Fig. 8A), while plasma urea and creatinine concentrations were significantly increased in the enalapril/spironolactone group versus the enalapril/aprocitentan or the vehicle groups (both $P < 0.0001$, Fig. 8, B and C). No significant change in kalemia was observed during the study in any group. Natremia was slightly but significantly decreased in both the combination versus vehicle groups on day 11 (Table 2).

Discussion

The objectives of this study were: 1) to characterize the pharmacology of aprocitentan in experimental models of hypertension with various levels of plasma renin activity and 2) to demonstrate the efficacy and safety of aprocitentan with a RAS blocker in the treatment of hypertension.

We quantified by telemetry the dose-response curves of aprocitentan after single and repeated doses in two different rat models of hypertension. In DOCA-salt rats, blockade of the ET system with the dual ETA/ETB ERA aprocitentan was more effective than inhibition of the RAS with an angiotensin II type-1 receptor antagonist. In contrast, the reverse effect was observed in SHRs. The relative potency of RAS or ET blockade on BP is coherent with the activation status of the RAS and ET system in these two models, which differ, among many other parameters, by the degree of salt-sensitivity, which is much more marked in DOCA-salt rats.

Vascular ET-1 expression is increased in hypertension, particularly salt-dependent hypertension, in animal models and humans (Ergul et al., 1996; Schiffrin, 1998b). In DOCA-salt rats but not SHRs, ET-1 content is increased in resistance vessels, suggesting a contribution of vascular ET-1 to elevated BP levels, which is confirmed by the differential response to ERAs in these models (Schiffrin, 1998a). In hypertensive patients, ET-1 plasma levels are inversely correlated with plasma renin activity (Eljovich et al., 2001). As a consequence, ET receptor blockade is more efficacious on BP in low-renin/salt-sensitive than in normal/high-renin conditions (Schiffrin, 1998a), whereas RAS blockers are more effective in normal/high-renin than in low-renin/salt-sensitive conditions. The use of telemetry in conscious animals to continuously record BP allows the detection of small changes in BP and the evaluation and comparison of areas under the curve versus time, instead of intermittent BP measurements obtained with the indirect tail-cuff method. Even though SHRs and DOCA-salt rats did not have similar levels of BP, which could potentially affect the pharmacological response to RAS blockers and ERAs, the difference in contribution of the renin angiotensin aldosterone and ET systems in these models remains the main driver when considering the response to different pharmacological classes. Previous studies showed that pharmacological or genetic manipulation of the renin angiotensin aldosterone and ET systems in these models remains the main driver when considering the response to different pharmacological classes. Previous studies showed that pharmacological or genetic manipulation of the renin angiotensin aldosterone and ET systems in these models remains the main driver when considering the response to different pharmacological classes. Previous studies showed that pharmacological or genetic manipulation of the renin angiotensin aldosterone and ET systems in these models remains the main driver when considering the response to different pharmacological classes.

Although the link between ET-1 and salt dependence/volume expansion is not fully understood, several findings suggest a strong association: salt administration in mice overexpressing endothelial ET-1 leads to an increase in BP, which is associated with microvascular endothelial dysfunction and remodeling (Amiri et al., 2010). Salt triggers ET-1 production by endothelial cells, leading to cell dysfunction and decreased endothelial nitric oxide synthase activity (Herrera and Garvin, 2005). Conversely, decreasing salt intake leads to a decrease in plasma ET-1 levels and correction of endothelial dysfunction in hypertensive patients (Ferri et al., 1998).
The increase in vascular ET-1 in salt-dependent animal models (Schiffrin, 1998b), which is associated with endothelial dysfunction, favors vasoconstriction and an increase in renal vascular resistance, leading to a decrease in sodium excretion and volume expansion. In addition, ET-1 stimulates aldosterone production by the adrenal glands via both ETA and ETB receptors (Rossi et al., 1994); hence, dual ETA/ETB blockade can reduce plasma aldosterone concentrations in clinical (Sütsch et al., 2000) or experimental situations in which aldosterone secretion is upregulated.

Blockade of ET receptors not only tackles a system involved in salt-dependent hypertension mediated by ETA and ETB receptors but may also mitigate the deleterious effects of ET-1, leading to end-organ damage. Aprocitentan, has an ETA/ETB inhibitory potency ratio of 1:16 (Iglarz et al., 2008), and is a dual ETA/ETB ERA that blocks both receptors in vitro and in vivo. The blockade of ETB receptors was confirmed in vivo by a dose-dependent increase in plasma ET-1, since endothelial ETB receptors are involved in ET-1 clearance (Löffler et al., 1993). Since aprocitentan is slightly more potent with regard to ETA than ETB, blockade of ETB in vivo implies blockade of ETA receptors. In many pathologic conditions, including hypertension, vasodilatory endothelial ETB receptors are no longer functional, while the upregulation of ETB receptors in the media of blood vessels contributes to vasoconstriction (Kakoki et al., 1999; Iglarz et al., 2015). Although ETB receptors contribute to vasodilation in healthy volunteers (Verhaar et al., 1998), this is not the case in patients with essential hypertension or insulin resistance. In these populations, ETB receptors can contribute to vasoconstriction since vasodilation in the forearm achieved with dual ETA/ETB blockade was superior to that achieved with ETA-selective agents (Cardillo et al., 1999; Shemyakin et al., 2006). In addition to vasoconstriction, ET-1 induces inflammation, fibrosis, and hyperplasia via the ETA and ETB receptors. These effects, and the ET system as a whole, may play a key role in end-organ damage. This hypothesis is supported by the upregulation of the ET system in pathologic situations, local autocrine/paracrine activity of the ET system, and the wide-ranging effects of this system on different body tissues (Iglarz and Clozel, 2010). As a consequence, blockade of both receptors improves vascular remodeling to a greater extent than ETA-selective blockade (Amiri et al., 2010), which prevents angiotensin II- and aldosterone-induced end-organ damage (Muller et al., 2000; Valero-Munoz et al., 2016) and also improves survival either alone (Mulder et al., 1997) or in combination with an angiotensin converting enzyme inhibitor (ACEI) in rats with heart failure (Iwanaga et al., 2001) or renal impairment (Benigni et al., 1996). ERAs have previously demonstrated efficacy on vascular function in
DOCA-salt rats by decreasing oxidative stress (Schiffrin, 1998a; Callera et al., 2003). Oral administration of aprocitentan led to a long-term decrease in BP in hypertensive rats. This is consistent with its long half-life, and shows it has the potential to block detrimental effects arising from ET-1 binding to both ETA and ETB receptors, i.e., vasoconstriction, hypertrophy, and fibrosis. Indeed, chronic administration of aprocitentan tended to dose dependently decrease cardiac hypertrophy in DOCA-salt rats and improve renal hemodynamics, suggesting its potential to protect end organs in a rat model of hypertension associated with volume expansion.

Our data demonstrate that aprocitentan, unlike spironolactone, acts synergistically with valsartan or enalapril to decrease BP in normal- and low-renin models. This suggests...
that ET receptor blockade can provide significant benefits when administered with existing drugs by neutralizing the effects of a pressor system, which is distinct from the renin angiotensin aldosterone axis and its effects on kidneys and vessels. Spironolactone, an MRA, acts downstream of ACEIs or ARBs and has an additive effect, whereas aprocitentan acts in parallel on the ET system and has a synergistic effect. The ET and renin angiotensin aldosterone systems interact as ET-1 and angiotensin II cooperate in promoting smooth muscle cell proliferation and cardiomyocyte hypertrophy (Rossi et al., 1999). Similar to angiotensin II, ET-1 potentiates vasoconstriction by increasing calcium sensitivity of the contractile apparatus (Iglarz et al., 2002). Therefore, blockade of such a potentiating effect could explain the synergism

![Fig. 6. Effect of single oral administration of spironolactone, valsartan, or a combination of both on mean arterial pressure in conscious SHRs, n = 6 per group (A and C), and DOCA-salt rats, n = 13 per group (B and D), equipped with telemetry. The gray areas represent the difference between the calculated additive effect and the measured effect of the combination on MAP. The predicted additive effect at each time point is the sum of the effects of the two drugs. Each time point represents the mean of five following consecutive hours of the point represented.](image1)

![Fig. 7. Effect of single oral administration of aprocitentan, amlodipine, or a combination of both on mean arterial pressure over time in conscious DOCA-salt rats (n = 8 to 9 per group) equipped with telemetry. Refer to Materials and Methods for doses. The gray areas represent the difference between the calculated additive effect and the measured effect of the combination on MAP. The predicted additive effect at each time point is the sum of the effects of the two drugs. Each time point represents the mean of five following consecutive hours of the point represented.](image2)
observed between aprocitentan and valsartan or enalapril on BP. Despite a limited effect of RAS blockers in the low-renin model (Supplemental Fig. 2), combination of aprocitentan with valsartan was synergistic, indicating that residual RAS activity in this model could still contribute to an elevation in BP by interacting with the ET system. Interestingly, the synergy observed with enalapril or valsartan occurred in normal (SHR) or low-renin (DOCA-salt) models, suggesting that the reduction in BP obtained may be effective independent of renin status. In contrast, the efficacy of spironolactone is renin dependent, as demonstrated by reduced efficacy in decreasing BP in patients with high plasma renin activity (Williams et al., 2015). The efficacy of aprocitentan when administered on top of the calcium channel blocker amlodipine in DOCA-salt rats supports the selection of a novel pathway for the treatment of hypertension.

A complete blockade of the RAS, using combinations of ACEIs, ARBs, and/or renin inhibitors, increases the rate of renal failure in patients with cardiovascular or renal diseases probably by inducing a cardio hypoperfusion state associated with activation of alternative vasodilatory mechanisms [e.g., bradykinin, angiotensin (1–7) accumulation, and angiotensin II type-2 receptor stimulation, and consequently nitric oxide release], and a depletion of the angiotensinogen pool (Azizi and Ménard, 2004; Richer-Giudicelli et al., 2004; Parving et al., 2012; Fried et al., 2013). When prescribed with ACEIs or ARBs, MRAs amplify the pharmacological blockade of the renin angiotensin aldosterone system and increase the risk of hyperkalemia. In patients with severe renal impairment, the combination of blockers of the renin angiotensin aldosterone system (ACEIs, ARBs, renin inhibitors, and MRAs) markedly increases the risk of hyperkalemia (Lazich and Bakris, 2014). ERAs may not carry this risk, which is linked to the mode of action of aldosterone on the kidney. Although we did not observe a change in kalemia in SHRs following a low-salt diet and receiving the combination spironolactone/enalapril, our data indicate that, unlike spironolactone, the administration of aprocitentan and enalapril further reduces BP but does not trigger acute renal insufficiency. Therefore, hypertensive patients intolerant to an MRA/RAS blocker combination under salt-restricted conditions or diuretic could benefit from a dual ERA such as aprocitentan. These data confirm the validity of our approach in targeting a novel pathway distinct from the renin angiotensin aldosterone system to minimize potential renal safety issues.

Other safety issues, mostly observed with ETA-selective ERAs, may be alleviated with dual ERAs. Secondary effects of ETA-selective antagonists, such as aggravation of cell proliferation (Hocher et al., 2003) or exaggerated fluid retention (Vercauteren et al., 2017), may be due to chronic stimulation of unantagonized ETB receptors during ET A blockade. The absence of reflex tachycardia secondary to BP reduction following aprocitentan treatment confirms previous observations with other dual, but not ETA-selective, ERAs, suggesting a buffering of autonomic reflexes and absence of reactive

### Table 2

Natremia and kalemia in sodium-depleted SHRs at days 0, 5 and 11

| Treatment       | Veh 6 | Ena 6 | Veh 11 | Ena 11 | Veh + Spiro 11 | Ena + Apro 11 |
|-----------------|-------|-------|--------|--------|---------------|--------------|
| Natremia (mmol/l) | 139 ± 1 | 140 ± 1 | 139 ± 1 | 139 ± 1 | 137 ± 1** | 138 ± 1* |
| Kalemia (mmol/l)  | 5.19 ± 0.17 | 4.69 ± 0.07 | 5.19 ± 0.12 | 5.19 ± 0.12 | 4.67 ± 0.10 | 4.65 ± 0.07 |

Ena, enalapril; Spiro, spironolactone; Veh, vehicle.

*P < 0.05; **P < 0.01 vs. vehicle group.
neurohormonal stimulation (Liu et al., 2001; Vercauteren et al., 2017).

In summary, our data provide a comprehensive rationale for developing the dual ETA/ETB ERA antagonist in hypertensive patients, especially those with volume-dependent hypertension. Blockade of ET receptors is an alternative to classic combinations of blockers of the renin angiotensin aldosterone system (i.e., RAS blockers plus MRA) with diuretics and/or calcium channel blockers. Since ET-1 is involved in many cellular functions, ETA/ETB receptor blockade could provide additional beneficial effects on endothelial function and catecholamine and aldosterone levels, and by minimizing end-organ damage, thereby complementing the protective effects of a long-term decrease in BP.

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