ET\textsubscript{A} RECEPTORS BLOCKADE, BY ACTIVATING ET\textsubscript{B} RECEPTORS, INCREASES VASCULAR PERMEABILITY AND INDUCES EXAGGERATED FLUID RETENTION.

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Abstract

Endothelin receptor antagonists have been associated with fluid retention. It has been suggested that, of the two endothelin receptor subtypes, ET<sub>B</sub> receptors should not be blocked, because of their involvement in natriuresis and diuresis. Surprisingly, clinical data suggest that ET<sub>A</sub>-selective antagonists pose a greater risk of fluid overload than dual antagonists. The purpose of this study was to evaluate the contribution of each endothelin receptor to fluid retention and vascular permeability in rats. Sitaxentan and ambrisentan as ET<sub>A</sub>-selective antagonists, bosentan and macitentan as dual antagonists were used as representatives of each class, respectively. ET<sub>A</sub>-selective antagonism caused dose-dependent haematocrit/haemoglobin decrease that was prevented by ET<sub>B</sub>-selective receptor antagonism. ET<sub>A</sub>-selective antagonism led to a significant blood pressure reduction, plasma volume expansion, and more vascular permeability increase than dual antagonism. Isolated vessels experiments showed that ET<sub>A</sub>-selective antagonism increased vascular permeability via ET<sub>B</sub> receptor overstimulation. Acutely, ET<sub>A</sub>-selective but not dual antagonism activated sympathetic activity and increased plasma arginine vasopressin and aldosterone concentrations. Haematocrit/haemoglobin decrease induced by ET<sub>A</sub>-selective antagonism was reduced in Brattleboro rats and in Wistar rats treated with an arginine vasopressin receptor antagonist. Finally haematocrit/haemoglobin decrease was larger in the venous than in the arterial side, suggesting fluid redistribution. In conclusion, endothelin receptor antagonists, and particularly ET<sub>A</sub>-selective antagonists, by activating ET<sub>B</sub> receptors, favour edema formation by causing 1) fluid retention resulting from arginine vasopressin and aldosterone activation secondary to vasodilation, 2) vascular permeability increase. Plasma volume redistribution may explain the clinical observation of haematocrit/haemoglobin decrease even in the absence of signs of fluid retention.
Introduction

Endothelin-1 (ET-1) may play a role in the pathophysiology of a number of diseases (Lerman et al., 1991; Luscher et al., 1993). Efforts to oppose its deleterious effects have led to the discovery of selective and dual antagonists of one or both of its receptors, ET\textsubscript{A} and ET\textsubscript{B}. Soon after the first clinical studies were initiated, it became clear that ET receptor antagonists (ERAs) can cause decreases in haematocrit (Hct) / haemoglobin (Hb) and signs of fluid retention. The decrease in Hb was clearly secondary to an increase in plasma volume since there were no signs of red blood cell changes, bone marrow depression, hemolysis, or bleeding. Signs of fluid retention are seen with virtually all ERAs and may be severe depending on the clinical context, the dose, and the degree of selectivity between ET\textsubscript{A} and ET\textsubscript{B} receptors of the different molecules (Battistini et al., 2006). In fact, drugs selective for the ET\textsubscript{A} receptor seem to cause a higher incidence and severity of fluid overload and edema rate than dual ERAs. In clinical studies, the ET\textsubscript{A}-selective antagonists darusentan in resistant hypertension (Black et al., 2007), zibotentan and atrasentan in prostate cancer (Carducci et al., 2007; James et al., 2009), avosentan in diabetic nephropathy (Mann et al., 2010), ambrisentan (Galie et al., 2008) and sitaxentan (Barst et al., 2004) in pulmonary arterial hypertension (PAH), induced edema or fluid overload in 12%, 38%, 28%, 15%, 18% and 8% of patients (placebo-corrected), respectively, and sometimes increased risk of heart failure and death (Luscher et al., 2002; Carducci et al., 2007; Mann et al., 2010). In the AMBITION trial in PAH patients, combination of ET\textsubscript{A}-selective ambrisentan with phosphodiesterase 5 inhibitor tadalafil led to a higher incidence of edema (45% in combination) than with tadalafil alone (28%) (Galie et al., 2015). In contrast, among dual ERAs, bosentan induced fluid retention in only 1.7% of PAH patients (placebo-corrected) (Rubin et al., 2002). The rate was higher in patients with chronic heart failure (Kaluski et al., 2008). Macitentan,
alone or in combination with phosphodiesterase 5 inhibitors, caused no increase in edema rate compared to placebo in the phase 3 trial SERAPHIN in PAH patients (Pulido et al., 2013).

A higher risk of edema with ET₄-selective receptor antagonists seems in contradiction with publications showing that ET₅-selective receptor antagonists or ET₅ knock-out cause fluid retention and edema (Gariepy et al., 2000; Ge et al., 2006). It is intriguing that both selective blockade of ET₅ receptors and selective blockade of ET₄ receptors, actually inhibit water and sodium excretion (Girchev et al., 1998; Nakano and Pollock, 2009; Boesen and Pollock, 2010). Interestingly, the fluid retention caused by ET₄- or ET₅-selective inhibition could be abolished by addition of the antagonist of the other, ET₅ or ET₄ receptor, respectively (Elmarakby et al., 2004; Ohkita et al., 2005; Kitada et al., 2009; Boesen and Pollock, 2010), showing that it was not the blockade of one receptor, but the overactivation of the other, that caused the phenotype of water or salt retention. It seems, therefore, that in both cases, selective blockade of one receptor subtype causes an imbalance and allows ET-1 - an ET₄/ET₅ agonist - to overactivate the non-antagonized receptor. This asymmetry of blockade can be assimilated to the phenomenon of cross-talk, by which a selective antagonism or selective receptor knock-out can be inefficient to block the action of the agonist because of the persistent route of signaling by the unblocked receptor. In tissues expressing both receptors, many examples of cross-talk have been reported with endothelin receptors (Clozel and Gray, 1995; Pollock, 2005; Sauvageau et al., 2007) probably due to ET₄/ET₅ receptors hetero-dimerization (Harada et al., 2002; Gregan et al., 2004). We hypothesized that this asymmetry of blockade between both receptors could contribute to the higher rates of fluid retention observed with ET₄-selective vis-à-vis dual ERAs in their respective clinical trials.
The present study was designed to establish an experimental model of fluid overload in response to ERAs, to investigate the contribution of each ET receptor subtype to its pathophysiology after acute and chronic administration of prototypic ERAs, sitaxentan and ambrisentan as representatives of the ET₄-selective antagonist class, and bosentan and macitentan as representatives of the dual receptor antagonist class. We found that ET₄-selective receptor antagonism led to vasodilation, arginine vasopressin (AVP) activation, reduction in water excretion and increased vascular leakage. Dual antagonism caused no vasodilation in these normal rats, no change in water excretion, no significant AVP increase and no vascular leakage.
Materials and Methods

Animals and drugs Original studies in animals have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, 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 housed in climate-controlled conditions with a 12-hour light/dark cycle. Wistar rats were obtained from Harlan Laboratories (AD Horst, Netherlands); homozygous Brattleboro rats lacking the AVP gene and rats of the background Long-Evans strain were obtained from the Rat Resource and Research Center (Columbia, Missouri). All experiments were performed in 8- to 12-week-old males rats. Compounds were administered in volumes of 5 mL/kg body weight. Endothelin receptor antagonists were synthesized by Actelion Pharmaceuticals Ltd.

Effect of acute administration of ERAs on fluid retention Because in humans the decrease in Hct and Hb observed with ERAs appears to be secondary to an increase in plasma volume, the change in these 2 variables was used as marker of fluid retention. Dose-response curves of sitaxentan (n=8 per dose) and bosentan (n=7 per dose), with doses ranging from 10 to 300 mg/kg for both drugs were performed, and the dose inducing the maximal decrease in Hct and Hb was selected for all acute experiments. Animals were assigned to groups in a stratified random manner according to their body weight and baseline Hct. A single oral dose of each drug or vehicle (gelatin) was administered by gavage and at 24 hours, sublingual blood was sampled under isoflurane-induced anesthesia (AttaneTM, MINRAD INC. Buffalo, New York). Hct, Hb and erythrocyte indices were measured using a hematology analyzer (Coulter AcT, Beckman Coulter, Nyon, Switzerland and Advia 2120i, Siemens Healthcare Diagnostics GmBH, Zurich,
Switzerland). Highest effective doses of sitaxentan (100 or 300 mg/kg) and bosentan (300 mg/kg) were selected for further studies. The selected dose of bosentan was demonstrated to block both ET\textsubscript{A} and ET\textsubscript{B} receptors \textit{in vivo} (Clozel et al., 1994).

**Role of ET\textsubscript{B} receptors in the mechanism of fluid retention** Prior to the experiment and under isoflurane-induced narcosis, the left jugular vein of rats was cannulated with a NaCl-heparin filled catheter. The catheter was then connected to a tether and harness attached to a swivel system equipment (Instech Laboratories Inc, Plymouth, USA). After a 2-day recovery period, conscious rats were infused either with vehicle (NaCl, 0.9\%) or BQ-788 (ET\textsubscript{B}-selective receptor antagonist, Tocris Bioscience, Abingdon, UK) for 6 hours, starting at the time of vehicle or sitaxentan administration (300 mg/kg, n=8 per group); 24 hours later, hematological variables were measured. The dose of 1 mg/kg/hour of BQ-788 was selected as it completely inhibited the ET\textsubscript{B}-dependent blood pressure decrease to ET-1 in conscious rats (Okada and Nishikibe, 2002).

**Effect of ERAs on hemodynamics in conscious rats** Animals were instrumented micro-surgically with a telemetry pressure transmitter implanted in the peritoneal cavity (Data Science International, Minnesota, USA) under isoflurane-induced narcosis. In brief, the pressure catheter was inserted into the aorta, below the renal artery pointing upstream. The abdomen was closed and the transmitter sutured to the abdominal musculature. Blood pressure was collected continuously using the Dataquest ART Gold acquisition system (version 3.01). Systolic, mean and diastolic arterial pressures, and heart rate were collected at 5-minute intervals. Data are shown as 24-hour means. Sitaxentan 100 mg/kg/day, bosentan 300 mg/kg/day, or vehicle were administered by gavage for 14 days (n=4-5 per group).
Effect of ERAs on plasma volume, arterio-venous gradient and vascular permeability

Sitaxentan (100 mg/kg/day), bosentan (300 mg/kg/day), or vehicle were administered for 14 days to Wistar rats (n=12 per group). Body weight was measured daily. Sublingual blood sampling for Hct/Hb measurement was performed at baseline, day 7 and 14 post administration in 7 to 8 animals per group. At the end of the treatment phase, all rats were anesthetized by isoflurane and placed on a thermostatically controlled heating table to maintain body temperature (37-38°C). A polyethylene conductance catheter was inserted in the femoral artery and advanced into the aorta. In parallel, a second catheter was introduced into the femoral vein and advanced into the vena cava. Measurements were performed for 10 minutes using a PowerLab data acquisition system (IOX 2.8.0.14 Data acquisition; Emka Technologies, Paris, France) connected to a computer equipped with Datanalist software (v2.6.1.12; Emka Technologies). Calibration of the conductance catheter and blood pressure transducer were performed before each measurement. At the end of the recording, arterial and venous blood was collected for arterio-venous Hct/Hb measurement (as described above) and 6 rats per group were submitted to a terminal protocol for determination of plasma volume and vascular permeability using Evans blue as previously described (Astrand and Saltin, 1964; Dallal and Chang, 1994).

Effects of ERAs on glomerular function, renal blood flow and free water clearance

Terminal renal clearance was assessed after a 14-day period treatment with sitaxentan (100 mg/kg/day), bosentan (300 mg/kg/day), or vehicle (n=9 per groups). Briefly, rats were anesthetized with 100 mg/kg Inactin, placed on a thermostatically controlled heating table, and cannulated for tracheotomy, right femoral vein for inulin and p-aminohippurate (PAH) infusion, right femoral artery for blood sampling and hemodynamics measurements (mean arterial blood pressure and heart rate), and the bladder to collect urine at 2 different time points after the
stabilization period. Urine and plasma were analyzed for inulin and PAH concentrations as described previously (Ding et al., 2003). These measurements allowed the assessment of glomerular filtration rate (GFR), renal plasma flow (RPF), renal vascular resistance (RVR) and the glomerular filtration fraction (FF). GFR and RPF were reported as ml per min and per 100 g of body weight. Results of the 2 clearance periods were averaged. Acutely, renal excretory function was evaluated at baseline and 6 hours after single oral administration of vehicle, bosentan or sitaxentan (both 300 mg/kg, n=8 per group). A water load (20 mL/kg tap water) was administered orally by gavage and rats were placed in metabolic cages for 3 hours for urine collection; a blood sample was taken 9 hours after administration of vehicle, bosentan, or sitaxentan. Urine (U) and plasma (P) osmolality were determined by using an osmometer (Löser Messtechnik, Berlin, Germany). Electrolyte concentrations and creatinine were measured using a Synchron analyzer (CX-5, Beckman Coulter, Nyon, Switzerland) and the free water clearance was calculated.

**Acute effects of ERAs on sympathetic activity** Prior to the experiment and under isoflurane-induced narcosis, the left jugular vein of rats was cannulated (see above). The swivel system equipment was allowing minimal disruption to the animal during blood sampling. After a 2-day recovery period, rats were orally treated with either sitaxentan (300 mg/kg, n=6), bosentan (300 mg/kg, n=9) or vehicle (gelatin, n=7). Blood was sampled at baseline, 6 and 24 hours post-oral treatment for plasma norepinephrine concentration quantification by radioimmunoassay (IBL international, Hamburg, Germany). Two animals in the vehicle group and 3 in the sitaxentan group could not be properly sampled due to catheter clotting.

**Acute effects of ERAs on arginine vasopressin pathway** Six and 24 hours after single oral administration of bosentan and sitaxentan (300 mg/kg for both, n=7 per group), Wistar rats were
decapitated without prior anesthesia, trunk blood was collected, and plasma AVP and serum aldosterone concentrations were determined by radioimmunoassay (Bühlman Laboratories AG, Schönenbuch, Switzerland) and ELISA kit (IBL, Hamburg, Germany), respectively. Kidneys were harvested, fixed, embedded, and sliced in 4 µm sections. The tissue expression of aquaporin 2 (AQP2) water channels was assessed as the intensity of immune-staining, using a rabbit polyclonal anti-rat antibody raised against AQP2 (Abcam, Cambridge, UK). Immune labeling was evaluated with a multi-observer, bright-field microscope. In order to confirm the role of AVP in fluid retention, sitaxentan was then administrated to AVP-deficient Brattleboro rats and their Long Evans controls (n=7 per group). Sitaxentan (300 mg/kg) was also administered to Wistar rats treated orally by gavage with tolvaptan (selective AVPV2 receptor antagonist, Otsuka Pharmaceuticals Ltd., Tokyo, Japan) at a dose of 10 mg/kg or vehicle (gelatin) (n=12-13 per group). After 24 hours, blood was sampled for hematology measurements in both Brattleboro rats or tolvaptan-treated Wistar rats.

**Role of ET\textsubscript{B} receptors in vascular leakage ex vivo** Thoracic aortic rings 10 mm in length were dissected in cold Krebs (4°C) in a petri dish. Intravascular blood was gently removed and collaterals were sealed. Rings were then placed in an organ bath in presence of Krebs (37°C). After a 30-minute resting period, pharmacological agents or their vehicle (DMSO) were added to the Krebs for 30 minutes. After incubation with ET\textsubscript{B}-selective receptor agonist sarafotoxin S6c (1 nM, Sigma, Buchs, Switzerland) or ET\textsubscript{A}-selective ERAs ambrisentan/sitaxentan, (100 nM) or dual ERAs macitentan/bosentan (100 nM) in presence or absence of a ET\textsubscript{B}-selective receptor blocker BQ-788 (1 nM, Sigma, Buchs, Switzerland), nitric oxide (NO) synthase inhibitor L-NAME (0.1 mM, Sigma, Buchs, Switzerland) or VEGF receptor blocker SUGEN5416 (SU5416, 1 µmol/l, Sigma, Buchs, Switzerland), aortic rings were cannulated between two catheters.
connected to 21G needles in a Petri dish filled with warm Krebs. The receptor selectivity profile of ERAs was confirmed by assessing their ability to inhibit sarafotoxin (S6c)-induced vascular leakage (Supplementary Figure S1A). Perfused vessels were gently infused with 0.8 mL of Evans blue solution (0.5 mg/mL) for 1 minute. Vessels were gently flushed for 30 seconds with Krebs, then removed from the system and cut longitudinally. After overnight drying in an oven (60°C), tissues were put in 150 µL formamide and incubated for 24 hours at 37°C. Evans Blue concentration was measured by spectrophotometry (wavelengths 620 and 740 nM). Vascular Evans blue quantification in isolated vessels is considered to reflect the binding of the dye on the internal lamina and allows to monitor the direct effect of drug tested on vascular permeability.

**Statistical analyses** The statistical comparison between the dose-response effects of sitaxentan and bosentan on Hct/Hb reduction was performed by using a one-way ANOVA, followed by a Dunnett multiple comparison test. Statistical comparison between multiple groups was performed using one or two-way ANOVA, with repeated measures when applicable, followed by a Newman Keuls or Tukey’s multiple comparisons post hoc tests. Unpaired t-tests were performed to analyze the effect of sitaxentan treatment in Brattleboro rats, combined with tolvaptan or BQ-788 vs. sitaxentan in Long Evans rats. Unpaired t-tests were also performed to analyze the effect of sitaxentan and bosentan on blood pressure and heart rate, by comparing area between curves. Analyses were performed using Prism software (GraphPad Prism 5). Differences were considered significant at $P < 0.05$. 
Results

ERAs with different selectivity profiles have differential acute effects on fluid retention: role of ET$_B$ receptors

Bosentan had small non-statistically significant effects on Hct up to the highest dose of 300 mg/kg (Figure 1A). Sitaxentan induced a significant dose-dependent decrease in Hct and Hb at doses of 30 mg/kg and above with a maximal effect at 300 mg/kg (Figure 1B). To test the hypothesis that the ET$_B$ receptor subtype could be responsible for the Hct reduction seen after a treatment with the ET$_A$-selective antagonist, the ET$_B$-selective antagonist BQ-788 was co-administered. BQ-788 itself had no significant effect on Hct (change from baseline 0.3 ± 0.9 vs. -0.5 ± 0.5 % after vehicle) but significantly inhibited the effect of sitaxentan on Hct change from baseline (Figure 1C).

ERAs have differential chronic effects on fluid retention, fluid redistribution, and vascular permeability

Sitaxentan (100 mg/kg/day) induced a non-significant increase in body weight, a significant decrease in Hct/Hb (by 7% and 7.5%, respectively) and a significant plasma volume expansion as measured by Evans blue (+29%), when compared to vehicle at day 14 (Figure 2). In contrast, bosentan (300 mg/kg/day) had no overall effect on body weight nor on plasma volume (+12%) compared to vehicle. Bosentan however significantly decreased Hct and Hb (by 2.5% and 2%, respectively) compared to vehicle at 14 days - although significantly less than sitaxentan (Figure 2). No changes in erythrocyte indices were noted with any compound, confirming that the reductions in Hct/Hb values were not due to direct effects on red blood cells but to plasma volume changes (Table 1). In order to understand the decrease in venous Hct/Hb by bosentan in the absence of effect on erythrocyte indices and despite no significant effect on plasma volume,
arterial and venous Hct/Hb were concomitantly measured after 14 days of treatment with both compounds. Sitaxentan induced a significant arterio-venous gradient of hemodilution, with a greater decrease in Hb and Hct (by 11.5% and 9%, respectively) in the vein that in the arterial compartment (7 and 4%, respectively) (Figure 3). Bosentan decreased venous Hb and Hct (by 3% and 1.5%, respectively) but slightly affected arterial Hb and Hct (-0.8% and -0.4%, respectively). Finally, we assessed vascular permeability in vivo. Sitaxentan significantly increased Evans blue distribution in the kidney and non-significantly in heart, aorta, and lungs. Bosentan had no significant effect (Figure 4). Based on the observation that chronic ET_A-selective receptor blockade tended to increase vascular permeability in vivo, we performed experiments on isolated vessels to confirm these findings. ET_A-selective antagonists sitaxentan and ambrisentan, but not dual receptor antagonists bosentan and macitentan, induced vascular leakage (Figure 5A and supplementary Figure S1B). Vascular leakage induced by ambrisentan was reduced by the NO synthase blocker L-NAME, and by the VEGF receptor blocker SUGEN5416 (Figure 5B,C). Furthermore, presence of the ET_B receptor blocker BQ-788 reduced the increase of vascular leakage induced by ambrisentan (Figure 5D). We then confirmed the involvement of unblocked ET_B receptors by showing that direct ET_B receptors stimulation with sarafotoxin S6c (1 nM) induced vascular leakage. This effect, as observed with ambrisentan, was prevented in presence of NO synthase and VEGF receptor blockers (Figure 5E,F). Therefore, selective blockade of ET_A receptors triggers an overstimulation of the unblocked ET_B receptors by endogenous ET-1, leading to increased vascular leakage via VEGF/NO production.
ERAs have differential effects on kidney function and hemodynamics

We then evaluated the mechanisms that could be implicated in the observed fluid retention: involvement of the kidney and/or hemodynamic changes. In an acute experiment using a water load protocol, sitaxentan led to a significant reduction in urine volume and CH$_2$O together with an increase in urine osmolality and a decreased plasma osmolality compared to vehicle (Figure 6) (for baseline values see the supplementary Table S1). Sitaxentan increased chloride fractional excretion and decreased potassium excretion but did not change the excretion of sodium. Creatinine clearance was not affected by sitaxentan (Table 2). Bosentan did not affect free water clearance, fractional ions excretion or creatinine clearance. Chronic treatment with sitaxentan did not alter GFR, RPF, RVR or FF versus vehicle (Figure 7). No change in plasma or urine sodium was observed (data not shown). In contrast to sitaxentan, bosentan increased GFR and FF as compared with vehicle control rats (Figure 7 A,D).

The assessment of hemodynamic impact of ERAs was performed on conscious rats implanted with telemetry devices. Sitaxentan induced a sustained decrease in systemic blood pressure associated with an increase in heart rate over the 14 days of treatment. Bosentan had no hemodynamic effect in these healthy rats (Figure 8).

The fluid retention induced by ET$_A$-selective receptor antagonists involves arginine vasopressin pathway

Based on the findings of decreased free water clearance with sitaxentan, and of plasma volume expansion, we studied the effect of acute administration of sitaxentan and bosentan on the antidiuretic hormone AVP and other hormones concentrations. Six hours after oral administration, sitaxentan, but not bosentan, induced a transient increase in norepinephrine and a three-fold increase in plasma AVP concentration compared with vehicle (Figure 9A,B). Serum aldosterone
was also increased by sitaxentan, but not by bosentan (Figure 9C). Norepinephrine, AVP and aldosterone returned to baseline concentrations at 24 hours. A decrease in Hct was significant for sitaxentan at 24 hours (-5%) but not for bosentan (-3%) (supplementary Table S2). Histological analysis of inner medulla collecting duct (IMCD) of the kidney showed that at 6 hours, sitaxentan increased the staining of the water channels regulated by AVP, aquaporin-2 (AQP2), compared with vehicle-treated rats, whereas bosentan had less pronounced effect (Figure 10A).

To assess the relationship between increased AVP and fluid overload induced by sitaxentan, we used the AVP-lacking Brattleboro rat and its control strain, Long Evans rat. Brattleboro rats exhibited at baseline lower Hct values as compared to Long Evans rats (43.6 ± 0.3 vs. 46.3 ± 0.2 % respectively; P < 0.001). The decrease in Hct induced by single-dose sitaxentan was significantly blunted in Brattleboro compared to control Long Evans rats (Figure 10B). We then evaluated the effect of selective blockade of the AVP V2 receptors using tolvaptan 10 mg/kg (Miyazaki et al., 2005), on the sitaxentan-induced decrease in Hct in Wistar rats. While tolvaptan itself had no significant effect on Hct (change from baseline 0.2 ± 0.3 vs. 0.5 ± 0.4 % after vehicle; P=0.55), it fully prevented the sitaxentan-induced Hct decrease (Figure 10C).
Discussion

Fluid retention and edema are common adverse effects of ERAs. The incidence and severity of fluid retention vary among different ERAs and seem correlated to the degree of selectivity between ET\textsubscript{A} and ET\textsubscript{B} receptors. Selectivity of ERAs is best assessed \textit{in vivo}, as not only receptor affinities, but also other compound characteristics such as local concentrations and metabolites could impact their pharmacology. The purpose of the present study was to develop a model of ERAs-induced fluid retention in non-diseased rats, in order to exclude the influence of disease state on the development of fluid retention. Our data show that ET\textsubscript{A}-selective antagonism leads to systemic vasodilation, transient norepinephrine and AVP release, water retention and vascular leakage. Contrariwise, dual antagonism, either with bosentan or after addition of an ET\textsubscript{B}-selective antagonist to sitaxentan, has smaller effects on all variables.

Our data show that bosentan decreased Hct/Hb, without major signs of fluid retention. Conversely, sitaxentan decreased Hct/Hb and led to fluid retention, characterized by plasma volume expansion and increased body weight, fluid redistribution as shown by a much larger decrease in venous than arterial Hct/Hb, and increased vascular permeability. For both compounds, the magnitude of venous Hct/Hb decrease was comparable to clinical observations (Battistini et al., 2006). However, the greater Hct/Hb decrease in the venous than in the arterial compartment, suggesting that ERAs can induce significant fluid redistribution from artery to vein, has not been described in humans. In our study, bosentan had almost no effect on Hct/Hb on the arterial side. It would be interesting to study whether a dual antagonist like bosentan or macitentan would have any effect on arterial Hct/Hb in humans.

Our results, by showing that ET\textsubscript{A}-selective antagonism unlike dual receptor antagonism reduces blood pressure in healthy rats, are in line with clinical observations describing blood flow...
increase and blood pressure decrease with ET\textsubscript{A}-selective antagonism (Verhaar et al., 1998), whereas dual ERAs bosentan and macitentan had no effect on blood pressure in healthy volunteers, even at much higher doses than those efficacious in PAH (Sidharta et al., 2013). The effect of the ET\textsubscript{A}-selective antagonist can be explained by an overstimulation of the unblocked endothelial ET\textsubscript{B} receptors, as blockade of ET\textsubscript{B} receptors partially prevented forearm blood flow increase induced by ET\textsubscript{A}-selective antagonism (Verhaar et al., 1998), and by NO release. This vasodilation could lead to an early activation of baroreceptors, as confirmed in our studies by the heart rate increase, and to a rise in circulating norepinephrine, concomitant to an activation of the AVP system and a plasma aldosterone increase in sitaxentan-treated rats.

Our data point out a role of AVP in the pathogenesis of fluid retention. Sitaxentan induced a transient increase in plasma AVP concentrations and in renal AQP2 staining. The transient increase in circulating AVP observed at 6 hours may be apparent and could be masked at 24 hours by the increase in plasma volume secondary to sitaxentan treatment. This plasma volume expansion could also explain the absence of change of electrolyte profile. Alternatively, adaptive mechanisms such as attenuation of AVP signaling or secondary down-regulation of AVP receptors could have contributed to the maintenance of normal plasma sodium levels despite increased AVP concentration (Girchev et al., 1998; Oiso et al., 2003). Lastly, a compensatory role of natriuretic peptides -not measured in our studies- cannot be ruled out.

Overall, we report three independent observations supporting the role of AVP in the fluid retention induced by ET\textsubscript{A}-selective antagonism: 1) the decrease in free water clearance; 2) the increase in plasma AVP concentration and renal AQP2 staining; and 3) the blunting of sitaxentan-induced Hct/Hb decrease in Brattleboro rats and after tolvaptan treatment. Our findings are in line with prior studies describing anti-diuretic and anti-natriuretic effects of ET\textsubscript{A}-
selective antagonist BQ-123 in Long Evans rats (Girchev et al., 1998); and absence of ambrisentan-induced fluid overload in mice with collecting duct-specific ET\textsubscript{A} receptor knockout (Stuart et al., 2013). Of interest, collecting duct-specific ET\textsubscript{A} receptor knockout causes increased plasma AVP concentrations (Ge et al., 2005), and the magnitude of this increase is similar to the amplitude observed with the ET\textsubscript{A}-selective antagonist sitaxentan. Beyond the response to hemodynamic changes, we cannot rule out a direct posterior pituitary gland stimulation of AVP secretion by ET\textsubscript{B} receptors activation resulting from ET\textsubscript{A} receptor blockade (Rossi, 2004) (not explored in the present study).

Our data also provide evidence of a cross-talk between ET receptors. As ET-1 is an agonist common to both receptors, endogenous ET-1 can fully activate the unblocked receptor when one receptor is antagonized, and either prevent efficacy of the selective antagonist on tissues where both receptors co-exist (Clozel and Gray, 1995; Sato and Amemiya, 1995; Fukuroda et al., 1996; Mickley et al., 1997), or cause adverse effects, such as fluid retention, paradoxical vasoconstriction (Kusmic et al., 2006) or increased fibrosis (Hocher et al., 2000). ET\textsubscript{A}/ET\textsubscript{B} hetero-dimerization is believed to play an important role in the phenomenon of redundancy or cross-talk (Harada et al., 2002; Gregan et al., 2004). In the collecting duct, ET-1 increases water excretion and maintains the pressure-natriuresis relationship via inhibition of tubular sodium reabsorption (Tomita et al., 1993; Ahn et al., 2004) mainly via activation of ET\textsubscript{B} receptors (Kohan, 1997; Gariepy et al., 2000; Ge et al., 2006). The role of ET\textsubscript{A} receptors in the renal medulla and the collecting duct has been much less studied and is still debated, but several studies (Girchev et al., 1998; Nakano and Pollock, 2009; Boesen and Pollock, 2010) but not all (Kohan, 2006) suggest that ET\textsubscript{A} receptors may play the same role as that of ET\textsubscript{B} receptors in diuresis and natriuresis. One could expect that dual blockade will cause enhanced water and
sodium retention compared to single receptor blockade. However, the contrary happens: although either ET\textsubscript{A} or ET\textsubscript{B}-selective receptor blockade can cause water retention, this can be reduced, but not aggravated, by dual blockade of the two receptors, as in our study: 1) ET\textsubscript{A}-selective receptor antagonism, and less dual blockade, decreased water excretion and free water clearance; 2) the decrease in Hct/Hb caused by sitaxentan could be reduced by addition of ET\textsubscript{B} receptor blocker BQ-788. It had been hypothesized that, at high doses of ET\textsubscript{A} receptor antagonists, a loss of selectivity could be responsible for triggering edema. However, the high selectivity of some of these compounds (e.g. avosentan and zibotentan) suggests that this is not the case. Overall, our findings indicate that in rats, stimulation of the unblocked ET\textsubscript{B} receptors in presence of ET\textsubscript{A} receptor antagonist, but not functional antagonism of the ET\textsubscript{A} receptor \textit{per se}, can be detrimental, and that blockade of both receptors is less likely to cause water retention than single receptor blockade.

The observation of an acute and transient AVP activation resulting from ET\textsubscript{A}-selective blockade is in line with clinical observations reporting rapid development of body weight increase and water retention with avosentan (Smolander et al., 2009), water retention and heart failure with atrasentan (Carducci et al., 2007) and fluid retention with darusentan (Luscher et al., 2002).

Among the factors contributing to edema formation, we speculate that plasma volume expansion combined with increased vascular permeability could explain the observations obtained with ET\textsubscript{A}-selective antagonists. \textit{Ex vivo} experiments showed that ET\textsubscript{A}-selective blockade induced the same effect on vascular leakage via VEGF and NO production as ET\textsubscript{B} stimulation. These data confirm that ET\textsubscript{A}-selective blockade triggers an overstimulation of unblocked ET\textsubscript{B} receptors, leading to vascular leakage as the effect of ET\textsubscript{A}-selective blockade was abolished by ET\textsubscript{B} receptor antagonism. This finding is in line with the \textit{in vivo} observation that ET\textsubscript{A}-selective, but
not dual blockade, tended to increase vascular leakage. However, other mechanisms such as changes in hydrostatic or oncotic pressures could also occur in vivo. Endothelial ET<sub>B</sub> stimulation has been reported to induce NO-dependent relaxation and vascular extravasation. We show that ET<sub>B</sub> stimulation causes vascular leakage via NO/VEGF-dependent mechanisms. ET-1 can activate VEGF production via either ET<sub>A</sub> or ET<sub>B</sub> receptors (Pedram et al., 1997; Matsuura et al., 1998) and dual blockade prevented VEGF production and lung extravasation in rats exposed to viral infection (Carpenter et al., 2005) and in diabetic retinopathy (Iglarz et al., 2008). Our data reveal a detrimental role for endothelial ET<sub>B</sub> receptors and indicate that the selectivity profile of an ERA can influence vascular leakage, which is a key step in edema formation.

No head-to-head clinical study comparing the effects of ERAs on fluid retention has been performed. Several clinical studies with ET<sub>A</sub>-selective antagonists have resulted in mortality increases in relation to fluid retention issues, whereas this has not been observed with dual ERAs. Dual ERAs, however, in conditions of preexisting fluid retention or AVP increase, such as chronic heart failure or chronic renal failure, have caused significant fluid retention.

In conclusion, our results reveal the key role of ET<sub>B</sub> receptors overstimulation, triggered by ET<sub>A</sub> receptor antagonism, in fluid retention and vascular leakage. Extrapolated to humans, these observations could explain the more frequent fluid retention and edema rate seen with certain ERAs, particularly when combined with other vasodilators, or in situations of elevated AVP concentrations, such as congestive heart failure, aging, or renal dysfunction.
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Performed data analysis: Vercauteren, Strasser, Hess, Clozel, Trensz, Pasquali, Cattaneo, Iglarz

Wrote or contributed to the writing of the manuscript: Vercauteren, Iglarz, Clozel
References


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Figures Legends

Figure 1. Acute dose-response effect of (A) bosentan (n=7 per dose) and (B) sitaxentan (n=8 per dose) on haematocrit (Hct) and haemoglobin (Hb) 24 hours after a single oral administration. (C) Sitaxentan-induced decrease in Hct is reduced in rats infused with the ET$_B$ receptor blocker BQ-788 (1 mg/kg/hour) compared to rats treated with sitaxentan alone (300 mg/kg) (n=8/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle or sitaxentan alone. Values are means ± SEM.

Figure 2. Differential effect of a 14-day oral administration of bosentan (300 mg/kg/day) and sitaxentan (100 mg/kg/day) on fluid retention. (A) Body weight (n=12/group), (B) Hct (top) and Hb (bottom) (n=7-8/group), (C) Plasma volume at day 14 (n= 6/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. vehicle, ++$P < 0.01$, +++$P < 0.001$, ++++$P < 0.0001$ vs. bosentan. Values are means ± SEM.

Figure 3. Differential effect of a 14-day oral administration of bosentan (Bos.) and sitaxentan (Sitax.) on Hct and Hb in venous and arterial vascular beds vs. vehicle (Veh.). Sitaxentan (panel A, 100 mg/kg/day), compared to bosentan (panel B, 300 mg/kg/day), induces a greater decrease in the vein vs. arterial Hct/Hb, n=12/group. *$P < 0.05$ vs. vehicle. Values are means ± SEM.

Figure 4. Differential effect of a 14-day oral administration of bosentan (300 mg/kg/day) and sitaxentan (100 mg/kg/day) on Evans blue distribution in (A) heart, (B) lung, (C) aorta and (D) kidney. n= 6/group. *$P < 0.05$ vs. vehicle. Values are means ± SEM.
**Figure 5.** Effect of ERAs on vascular leakage. Unlike macitentan (Maci), ambrisentan (Ambri) increases (A) vascular leakage. This leakage was inhibited by (B) nitric oxide synthase inhibitor L-NAME, (C) VEGF blocker SUGEN5416 (SU5416) or (D) ET<sub>B</sub>-receptor blocker BQ-788. Like ambrisentan, ET<sub>B</sub>-selective agonist sarafotoxin S6c (S6c)-induced vascular leakage, which was also inhibited by either (E) L-NAME or (F) SU5416. *P < 0.05, ***P < 0.001 vs. CTL; +P < 0.05, ++P < 0.01, +++P < 0.001 vs. sarafotoxin S6c or ambrisentan, respectively. n=9-21/group. Values are means ± SEM.

**Figure 6.** Acute effect of ERAs on kidney water excretion function. Single oral administration of sitaxentan (300 mg/kg), but not bosentan (300 mg/kg), decreases (A) urine (U)- volume, (B) free water clearance (CH<sub>2</sub>O), (C) plasma (P)- osmolality and increases (D) urine (U)- osmolality. n=8/group. *P < 0.05, **P < 0.01 vs. vehicle; +P < 0.05, ++P < 0.01 vs. bosentan. Values are means ± SEM.

**Figure 7.** Effect of a 14-day oral administration of bosentan (300 mg/kg/day) and sitaxentan (100 mg/kg/day) on renal clearance parameter: (A) glomerular filtration rate (GFR), (B) renal plasma flow (RPF), (C) renal vascular resistance (RVR), and (D) filtration fraction (FF), (n=9/group). *P < 0.05, ****P < 0.0001 vs. vehicle; ++P < 0.01, ++++P < 0.0001 vs. bosentan. Values are means ± SEM.

**Figure 8.** Chronic effect of ERAs on hemodynamics obtained from conscious implanted rats with telemetry devices after a 14 days of oral administration. Compared to vehicle (n=4), sitaxentan (panel A, 100 mg/kg/day, n=5), but not bosentan (panel B, 300 mg/kg/day, n=5)
induced a decrease in mean arterial blood pressure over the 14 days of treatment associated with an increase in heart rate. **P < 0.01, ***P < 0.001 vs. vehicle. Values are 24-hour means ± SEM.

Figure 9. Effect of ERAs on norepinephrine, arginine vasopressin and aldosterone concentrations after a single oral administration. Sitaxentan (Sitax., 300 mg/kg, n=6-7), but not bosentan (Bos., 300 mg/kg, n=7-9) increases significantly (A) plasma norepinephrine, (B) plasma arginine vasopressin (AVP) and (C) serum aldosterone concentrations at 6 hours vs. vehicle (Veh., n=7). All hormones, returned to baseline concentrations at 24 hours after sitaxentan treatment. *P < 0.05, **P < 0.01 vs. vehicle at 6 hours; +P < 0.05, ++P < 0.01 vs. bosentan at 6 hours. Values are means ± SEM.

Figure 10. Effect of ERAs on AVP pathway after a single oral administration. Sitaxentan (300 mg/kg), but not bosentan (300 mg/kg) (A) increases aquaporin2 staining in the inner medulla collecting duct, at 6 hours after oral administration compared with vehicle, as shown by microscopic evaluation of transversal (T) and longitudinal (L) sections of the left renal medulla. Hct decrease induced by sitaxentan was abolished in (B) AVP-deprived Brattleboro (BRA) rats compared to their control Long Evans (LE) rats (n=7/group), and in (C) Wistar rats treated with tolvaptan (10 mg/kg), a selective AVP V2 receptor antagonist (n=12/group). *P < 0.05, ***P < 0.001 vs. sitaxentan alone. Values are means ± SEM.
Tables

Table 1 Effect of 14-day oral administration of bosentan (300 mg/kg/day) and sitaxentan (100 mg/kg/day) on erythrocyte indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). n=12/group. Values are means ± SEM.

<table>
<thead>
<tr>
<th>Erythrocytes indices</th>
<th>Vehicle</th>
<th>Bosentan</th>
<th>Sitaxentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV (fL)</td>
<td>55.5 ± 0.5</td>
<td>55.7 ± 0.3</td>
<td>55.7 ± 0.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.3 ± 0.2</td>
<td>17.1 ± 0.2</td>
<td>17.0 ± 0.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.3 ± 0.2</td>
<td>30.7 ± 0.2</td>
<td>30.4 ± 0.2</td>
</tr>
</tbody>
</table>
Table 2. Effect of acute oral administration of bosentan (300 mg/kg) and sitaxentan (300 mg/kg) on change from baseline in electrolytes fractional excretion and creatinine clearance. n= 8/group.

* P < 0.05, ** P < 0.01 vs. vehicle; + P < 0.05, ++ P < 0.01 vs. bosentan. Values are means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Bosentan</th>
<th>Sitaxentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional excretion (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>-0.04 ± 0.05</td>
<td>-0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Potassium</td>
<td>-0.95 ± 1.10</td>
<td>-0.13 ± 1.9</td>
<td>-7.6 ± 2.4*+</td>
</tr>
<tr>
<td>Chloride</td>
<td>-0.15 ± 0.04</td>
<td>-0.2 ± 0.1</td>
<td>0.4 ± 0.1**++,</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>0.07 ± 0.16</td>
<td>-0.02 ± 0.2</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9
**Figure 10**