Capturing the dynamics of systemic Renin-Angiotensin-Aldosterone System (RAAS) peptides heightens the understanding of the effect of benazepril in dogs

J. P. Mochel*, M. Peyrou†, M. Fink‡, G. Strehlau§, R. Mohamed†, J. M. Giraudel†, B. Ploeger** & M. Danhof**

*Department of Drug Metabolism and Pharmacokinetics, Novartis Campus St. Johann, Basel, Switzerland; †Department of Bioanalytics, Novartis Centre de Recherche Sante Animale SA, Aubin, Switzerland; ‡Department of Modeling and Simulation, Novartis Campus St. Johann, Basel, Switzerland; §Department of Drug Efficacy, Novartis Animal Health, Basel, Switzerland; †Department of Research and Development, Novartis Animal Health Australasia Pty Limited, Yarrandoo, NSW, Australia; **Department of Pharmacology, Leiden-Amsterdam Center for Drug Research, Leiden, The Netherlands

In dogs, activation of the Renin-Angiotensin-Aldosterone System (RAAS) is an important feature of congestive heart failure (CHF). Long-term increases in angiotensin II (AII) and aldosterone (ALD) lead to the progression of heart failure to its end stage. Angiotensin-converting enzyme inhibitors (ACEIs) are the foremost therapeutic option in the management of CHF. Recent literature has challenged the efficacy of ACEIs, based on modest reduction in urinary aldosterone (UALD) excretion despite marked inhibition of ACE activity. This study was designed to heighten the understanding of the effect of benazepril, a potent ACEI, on the RAAS, using a low-sodium diet as an experimental model of RAAS activation. Time course profiles of RAAS peptides and related areas under the curve (AUC24 hours) were used for comparison between benazepril and placebo groups. Results indicated substantial changes in the dynamics of these biomarkers. At presumed benazepril at steady state, significant differences in AUC24 hours of plasma renin activity (+90%), angiotensin I (+43%), and AII (53%) were found between benazepril and placebo-treated dogs. ALD decreased by 73% in plasma but only by 5% in urine. In conclusion, despite modest reduction in UALD excretion, benazepril markedly influences RAAS dynamics in dogs.

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Dr Jonathan P. Mochel, Novartis Campus St. Johann, WSJ-27.6, 4056 Basel, Switzerland. E-mail: jonathan.mochel@novartis.com

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INTRODUCTION

In dogs, congestive heart failure (CHF) most often develops consequent to chronic valvular heart disease (CVHD), also known as endocardiosis (Atkins et al., 2009). CVHD is a condition characterized by thickening and shortening of the atrioventricular valves, which affects about 75% of dogs over the age of 16 (Guglielmini, 2003). Similar to humans, activation of the Renin-Angiotensin-Aldosterone System (RAAS) (see Fig. 1) is one of the key neurohumoral responses to the reduced cardiac output observed in canine CHF (Watkins et al., 1976; Sayer et al., 2009). Over the past years, it has become evident that long-term increases in angiotensin II (AII) and aldosterone (ALD) contribute to an exaggerated workload, and a progressive remodeling of the heart (i.e., cardiac fibrosis), leading to the progression of heart failure to its end stage (Cohn et al., 2000; Shimizu et al., 2006). In patients with CHF, high AII and ALD concentrations have been determined as the predictors of increased mortality risk (Roig et al., 2000; Güder et al., 2007).

Angiotensin-converting enzyme inhibitors (ACEIs) are one of the lead therapeutic classes in the treatment for canine CHF. Benazepril (Fortekor®; Novartis Animal Health, Basel, Switzerland) is a potent ACEI with well-documented effectiveness in canine CHF (King et al., 1995; Lefebvre et al., 2007). In the BENCH Study (1999), dogs with mild to moderate CHF treated with benazepril lived on average 2.7 times longer (428 days), as compared with the placebo group (158 days). They also experienced a significant improvement in exercise tolerance and clinical condition after 28 days of therapy. Yet, recent literature has challenged ACEIs effectiveness in inhibiting the
RAAS. In a furosemide model of RAAS activation, Lantis and Atkins (2010) have shown that benazepril did not reduce UALD excretion despite marked inhibition of plasma ACE activity (ACEA). These findings emphasize the need for further research on the ability of ACEIs to effectively inhibit the RAAS in dogs. This manuscript presents the results of the first study on the dynamics of the entire renin-angiotensin cascade following oral administration of an ACE inhibitor, using a low-sodium diet as an experimental model of RAAS activation.

MATERIALS AND METHODS

The study was performed in compliance with a registered Swiss permit covering animal experiments for cardiovascular research in dogs as approved by the Cantonal Animal Welfare Committee. The study protocol was designed to use the fewest number of animals possible while being consistent with the scientific needs of the study, taking into account dedicated regulatory requirements.

Sixteen beagle dogs weighing between 12.0 and 19.5 kg (mean: 15.4 kg, standard deviation: 2.0 kg) were randomly allocated to two treatment groups: (i) benazepril 10 mg PO q24h (n: 8) and (ii) placebo (n: 8), respecting a homogeneous distribution of bodyweight and gender between groups. The median dosage of benazepril was 0.7 mg/kg. Suitability for inclusion was evaluated by a physical examination and confirmed by measuring selected hematological (RBC, WBC, Hb, Ht) and clinical chemistry (albumin, total protein, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine) parameters in blood specimens.

During the 1-week acclimation period, dogs were fed a regular diet (Biomill Adult Medium®, 0.5% sodium. Biomill SA., Herzogenbuchsee, Switzerland). They were fed a low-sodium diet [The low-sodium diet (LSD) has been validated in-house as a noninvasive, fully reversible, and reliable model of RAAS activation in dogs (data not shown)] (0.05% sodium) for 5 days thereafter, to attain a steady activation of the RAAS prior to the administration of the first test item, as reported by Kjolby et al. (2005). Drinking water quality was compliant with the Swiss Federal Regulation on Foodstuff and was offered ad libitum. Dogs were repeatedly sampled over 24-h periods after the first (day 1) and last (day 5) test item administration (see Fig. 2). Blood specimens were collected from the vena jugularis into 1.2 or 2.7 mL S-Monovette® tubes (Sarstedt Inc., Newton, NC, USA) for plasma renin activity (PRA), AI, AII, and ALD determination. Dogs were maintained in the same position (up and standing) during blood collection. Blood samples were cooled on ice immediately after withdrawal and centrifuged under refrigeration (2 ± 1 °C, 15 min) within 30 min of sampling. Plasma was then transferred into cooled polypropylene aliquots, snap-frozen, and stored at –80 °C before analysis. On the sampling days, dogs were housed in metabolism cages, after their bladder had been emptied by urinary catheterization. Urine was collected from the metabolism cages into cooled Erlenmeyer flasks, as described by Gardner et al. (2007), and pooled with (i) the residual urine collected by rinsing the cage receptacle with distilled water and (ii) the specimen collected by urinary catheterization at the end of the 24-h period. Urine samples were then transferred into two distinct plain tubes, for the determination of (i) UALD (stored at –80 °C), and (ii) potassium and sodium concentrations (stored at 4 °C), using an ion-selective electrode measurement method. Total UALD excretion (pg/24 h) was computed thereafter, using the volume of urine produced over 24 h. Ultimately, the renal clearance of aldosterone (CLR[ALD]) was derived from the ratio of total UALD excretion to the area under the curve of aldosterone plasma concentrations over 24 h. Aldosterone, potassium, and sodium

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**Fig. 1.** Schematic view of the systemic renin-angiotensin cascade. Angiotensinogen is converted into angiotensin I (AI) by the activated form of renin. Angiotensin II (AII) is derived from AI by enzymatic cleavage catalyzed by angiotensin-converting enzyme (ACE), although alternative, ACE-independent pathways can also lead to the production of AII. All stimulatory effect on aldosterone (ALD) release is mediated by type 1 Ang II receptors (AT1R). Compensatory mechanisms (e.g., AI-renin negative feedback loop) contribute to the regulation of renin release. Long-term increases in AII and ALD lead to end-organ damage and progression of heart failure. ACE inhibitors, like benazepril, act by preventing the formation of AII and the degradation of bradykinin, which acts as a potent vasodilator.

**Fig. 2.** Study outline. During the 1-week acclimation period, dogs were fed a regular diet. They were fed a low-sodium diet for 5 days thereafter, to attain a steady activation of the Renin-Angiotensin-Aldosterone System (RAAS). Blood and urine samples were collected over 24-h periods after the first (day 1) and last (day 5) test item administration.
fractional excretions (aldosterone fractional excretion (UfeALD), potassium fractional excretion (UfeK), and sodium fractional excretion (UfeNa)) were calculated using a clearance approach, as described by Lefebvre et al. (2008).

Plasma renin activity was determined by measuring the rate of AI formation after incubation of endogenous renin and angiotensinogen in plasma (2 h, 37 °C, pH 7.2). AI plasma concentrations were measured after liquid extraction using a validated enzyme immunoassay (EIA) test (S-1188 Angiotensin I-EIA kit; Host: Rabbit High Sensitivity CE-marked; Bachem, Bubendorf, Switzerland). Analyses were performed in duplicates, values with a CV% below 25% were retained for statistical evaluation. All plasma concentrations were analyzed using a validated EIA test with a specific monoclonal anti-AI antibody (A05880 Angiotensin II SPIE-IA kit; Bertin Pharma, Montigny le Bretonneux, France). Analyses were performed in duplicates, values with a CV% below 30% were retained for statistical evaluation (3% of all data were excluded based on this criterion). ALD and UALD concentrations were determined with a linear chromatography–tandem mass spectrometry method using an isotope dilution technique. Urine samples were not subjected to acid hydrolysis before extraction, that is, only free aldosterone concentrations were measured. Calibration standards ranging from 0.02 to 2.0 ng/mL and 0.05 to 10.0 ng/mL were used for quantification in plasma and urine matrices, respectively.

Systemic levels of RAAS peptides were expressed as percent change from baseline (herein referred to as the geometric mean of three successive predosing values), as follows:

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\text{Change from baseline (\%)} = 100 \times \frac{(\text{endpoint value}_n - \text{baseline})}{\text{baseline}}
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Results were analyzed by repeated measures analyses of variance (RMANOVA) including ‘treatment’ (benazepril vs. placebo), ‘time’, and ‘interaction between treatment and time’ as classification variables. Treatment groups were compared at each time point using linear contrasts.

Areas under the curve of the percent change from baseline, which are presented in Fig. 4. Compared to placebo, single and repeated administrations of benazepril resulted in highly significant increases \((P < 0.01)\) in PRA AUC24 hours (+71% and +90%, respectively), and in AI AUC24 hours (+43% for both sampling days). In benazepril-treated dogs, all AUC24 hours were decreased by 46% and 53% \((P < 0.05)\) on the first and the fifth administration day, respectively. No noticeable changes in ALD AUC24 hours were observed after the first administration. Benazepril triggered a 73% reduction in ALD AUC24 hours after the fifth administration, without reaching statistical significance.

The analysis of urinary data, as presented in Fig. 5, revealed a modest reduction in aldosterone elimination in benazepril-treated dogs. Differences from the placebo group seemed more pronounced after single than after multiple dosing (from \(-31\%\) to \(-5\%), \(-44\%\) to \(-29\%), and \(-17\%\) to \(+5\%), for UALD, UfeALD, and \(CL_{\text{CRE}}\) respectively). No differences in creatinine clearance \(CL_{\text{CRE}}\) could be found between treated groups.

Minor changes in urinary sodium elimination were noticed on both sampling days \((\text{ca. } -12\%\). In contrast to the first day where only slight differences were identified, a significant \((P < 0.05)\) decrease in potassium elimination \((\sim 32\%)\) was detected after the fifth administration. These findings were consistent with results from fractional excretions (UfeK \(-31\%).

DISCUSSION

In veterinary medicine, there is very little available information on the effect of ACEIs on the circulating levels of RAAS peptides. Knowlen et al. (1983) have reported nonsignificant differences
between pre- and postdose ALD values in seven CHF dogs chronically treated with captopril. Likewise, drops in ALD during intravenous captopril infusion for five consecutive days did not reach statistical significance in four healthy beagle dogs on a high-sodium diet (Boemke et al., 1995). In a study by Haggstrom et al. (1996), ALD and AII values decreased after 3 weeks of oral therapy with enalapril, yet only the reduction in ALD was statistically significant ($P < 0.05$). The current results provide the first description of the effect of an ACE inhibitor on the entire renin-angiotensin cascade in dogs.

**Benazepril effects systemic RAAS peptides**

Our data show that benazepril markedly influences the dynamics of systemic RAAS peptides, resulting in a substantial decrease in AII and ALD while increasing PRA and AI. These differences reflect the high level of regulation of the renin-angiotensin cascade. Increases in PRA and AI were triggered by the interruption of the AII-renin negative feedback loop, related to a decrease in AII in the benazepril group (Bussien et al., 1986; Geary et al., 1992; Steele et al., 2002).

Repeated administrations of benazepril also induced a marked reduction in ALD AUC$_{24}$ hours. The changes in ALD prompted a significant decrease in potassium urinary elimination and fractional excretion, but only modest differences in UALD excretion.

**Differences in urine vs. plasma aldosterone concentrations**

Recent literature indicated that the lesser effect of ACEIs on UALD compared to ALD could be related to extra-adrenal production of aldosterone. Xue and Siragy (2005) have for the first time reported the local expression of the aldosterone synthase CYP11B2 in the kidneys of normal rats. The authors were not able to detect aldosterone in the plasma of adrenalectomized rats, but found substantial amounts of aldosterone in the renal cortex. This pointed out that the locally produced aldosterone did not enter the systemic circulation and was

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Fig. 3. Dynamics of systemic Renin-Angiotensin-Aldosterone System (RAAS) peptides following single and repeated oral administrations of benazepril (10 mg PO, q24h) in healthy beagle dogs on a low-sodium diet. Results are expressed as percent change from baseline, where differences from the placebo group are presented at each time point. Vertical bars indicate one standard deviation (n: 8 per group). From top left to bottom right: plasma renin activity (PRA), AI, angiotensin II (AII), aldosterone (ALD). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The data support substantial differences in the dynamics of systemic RAAS peptides, resulting in elevated PRA and AI concentrations in the benazepril group, but decreased AII and ALD concentrations.
directly excreted in urine. As this pathway is mainly regulated by low-sodium intake, the use of a highly depleted sodium diet could have triggered the activation of both systemic and renal RAAS, resulting in an elevated UALD excretion in benazepril and placebo-treated dogs. However, this needs to be confirmed by additional experimental data. To date, the exact function of this local system, or its modulation by ACEIs, is not known (Siragy, 2006).

Another explanation could be that differences in urine vs. plasma concentrations were owing to changes in CL\textsubscript{R[ALD]}. From the relative increase in UALD and the drop in ALD AUC\textsubscript{24 hours} observed within the benazepril group between the first and the fifth administration follows an increase in CL\textsubscript{R[ALD]} (see Fig. 5). Because CL\textsubscript{CRE} was found to be almost constant, the changes in CL\textsubscript{R[ALD]} would be driven by shifts in tubular secretion/reabsorption (UfeALD). Unlike UALD, plasma ALD is robust against changes in CL\textsubscript{R[ALD]} because about 85% of aldosterone clearance takes place in the liver (Balikian, 1971).

Both scenarios suggest that ALD AUC\textsubscript{24 hours} is a more appropriate surrogate of aldosterone adrenal secretion than UALD. Finally, one should emphasize that the pharmacological effects of aldosterone are related to plasma, not urinary concentrations.

**Alternative pathways of AII production**

According to King et al. (1997), benazepril produces a long-lasting inhibition of ACEA, with an 85% inhibition at 24 h postadministration. Our results show that the effect of benazepril on AII fades out earlier with ca. 50% reduction in ALD AUC\textsubscript{24 hours}. This apparent disconnect could be related to the production of AII by the upregulation of ACE-independent pathways (see Fig. 1) (Balcells et al., 1997; Fyhrquist & Sajjonmaa, 2008) in response to renin and AI accumulation during short-term and long-term use of ACEIs (Geary et al., 1992). Van de Wal et al. (2006) have established that up to half of patients treated with an ACEI had high AII plasma concentrations despite low plasma ACEA. The incomplete reduction in AII in ALD may also have been the result of experiencing only partial blockade of ACEA because of an inadequate dose of ACEI. While the maximum recommended dose of benazepril was used in this experiment, this may not have been sufficient to offset the LSD-induced RAAS activation (Van de Wal et al., 2006) or to account for tissue ACEA (Swedberg et al., 1990).

**Clinical relevance**

Only few studies have investigated the relationship between AII and ALD exposures to increased morbidity/mortality risk in CHF dogs. ALD plasma concentrations were documented in dogs with CHF of diverse status (Knowlen et al., 1983). It was concluded that the increase in ALD was directly related to the clinical status of the patient.

In human cardiology, a rich body of literature has established the close relationship between AII/ALD concentrations and survival in patients with heart diseases. Swedberg et al. (1990) have found a significant and positive correlation between mortality and the concentrations of AII and ALD in a group of
severe untreated patients with CHF. In a 12-month follow-up study, increased AII plasma concentrations were a significant predictor of death or new heart failure episodes in patients with left ventricular dysfunction (Roig et al., 2000). More recently, Güder et al. (2007) have shown that high ALD plasma concentrations were a predictor of increased mortality risk that provided complementary prognostic value in patients with CHF of any cause and severity.

Limitations

Because of the small study size, the statistical significance of certain findings was hampered by low statistical power. This is illustrated by the nonsignificance of the 73% decrease in ALD AUC24 hours found in the benazepril group. Using back calculation, the power associated with this number was only of 38%.

Data were collected during a relatively short experimental time interval leaving it unclear whether additional administrations of benazepril (as seen in long-term therapeutic strategies) would have resulted in greater reductions of AII and ALD.

To better characterize the relationship between ACEA and AII, direct information on ACEA would have been necessary. This could not be performed because drawing extra samples for ACEA measurement would have resulted in exceeding the volume of withdrawn blood authorized in the aforementioned Swiss permit.

CONCLUSIONS

Despite the only modest reduction in UALD excretion, benazepril markedly influences RAAS dynamics in dogs, resulting in a substantial decrease in AII and ALD while increasing PRA and AI.

The effect of benazepril on AII and ALD evidenced in this study may be the driver of increased survival and improved quality of life in benazepril-treated dogs, similar to the findings in patients with CHF. To support and consolidate this hypothesis, systemic concentrations of AII and ALD in ACEI-treated vs. untreated dogs with spontaneous CHF should be investigated. If such a link can be established, profiling of these peptides could support the determination of the severity of heart failure, complement clinical and echocardiographic findings, and be used for therapeutic drug monitoring purposes.

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REFERENCES


