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The vasopeptidase inhibitor AVE7688 ameliorates Type 2 diabetic nephropathy

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Abstract

Aim/hypothesis. Pharmacological inhibition of the renin angiotensin system has proven clinical efficacy in nephropathies of various origins, including diabetic nephropathy. We tested the effects of the dual inhibition of both angiotensin converting enzyme and neutral endopeptidase by the vasopeptidase inhibitor AVE7688 in an animal model of Type 2 diabetic nephropathy.
Methods. We treated 56 obese Zucker diabetic fatty (ZDF, Gmi-fa/fa) rats aged 34-weeks with either placebo (n=9) or the vasopeptidase inhibitor AVE7688 in four different doses (each n=9; 3, 10, 30, or 60 mg/kg/d in chow). We used 11 heterozygous (+/fa) rats which received placebo and served as non-diabetic, lean controls. Urinary albumin/creatinine ratio was assessed as a marker of nephropathy at baseline (age 34-weeks) and after 10 weeks of chronic treatment.
Results. All obese animals had established diabetes mellitus that was not influenced by AVE7688 (HbA_{1c} >12%, stable in all dose groups). There was massive albuminuria in the homozygous ZDF rats (albumine/creatinine ratio >20 mg/mg vs minimal albuminuria in lean controls) that was decreased by AVE7688 in a dose dependent manner (Placebo 2.0±4.4 vs 11.9±1.8, 13.4±0.7, 13.6±2.8, and 19.8±2.8 mg/mg in the 3, 10, 30, and 60 mg/kg/d groups, respectively; all treatment groups p<0.05 vs Placebo).
Conclusion/interpretation. AVE7688 ameliorates proteinuria in Zucker diabetic fatty rats with established diabetes mellitus. Vasopeptidase inhibition represents an effective novel therapeutic principle for intervention in Type 2 diabetic nephropathy independent of metabolic control. [Diabetologia (2004) 47:98–103]

Keywords Zucker diabetic fatty rat · diabetes · microvascular complications · diabetic nephropathy · albuminuria · angiotensin converting enzyme · neutral endopeptidase · vasopeptidase inhibition · AVE7688

As a result of its dramatically increasing incidence, Type 2 diabetes has become the leading single cause of end stage renal disease in most industrialized countries [1]. Urinary excretion of albumin has been established as a sensitive marker of the degree of diabetic kidney damage, and the extent of albuminuria reflects the risk of cardiovascular end points as well as future incidence of end stage renal failure [2]. Pharmacological blockade of the renin angiotensin aldosterone system by either inhibition of angiotensin converting enzyme (ACE) or blockade of the angiotensin II receptor has clinically proven its ability to delay the progression of diabetic nephropathy [3, 4, 5, 6, 7, 8]. The novel vasopeptidase inhibitors could be of therapeutic value in a variety of nephropathies, based on their potential to increase tissue concentrations of bradykinin and the organoprotective natriuretic peptides through inhibition of neutral endopeptidase in addition

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Abbreviations: ACE, angiotensin converting enzyme; ZDF, Zucker diabetic fatty.

to ACE [9]. Indeed, the vasopeptidase inhibitor omapatrilat has shown potent nephroprotection in models of subtotal rat nephrectomy [10, 11] and in a hypertensive, hypo-insulinaemic nephropathy model [12]. However, the potential benefit of pharmacological intervention with a vasopeptidase inhibition in insulin resistant, Type 2 diabetic nephropathy has not been evaluated so far.

In this study, we therefore tested whether chronic treatment with AVE7688, a novel vasopeptidase inhibitor, can effectively reduce urinary albumin excretion, an established biomarker of renal dysfunction, in diabetic nephropathy. We used the Zucker diabetic fatty (ZDF) rat as a suitable model because it is characterized by obesity, insulin resistance, Type 2 diabetes, and progressive proteinuria.

Materials and methods

Compounds. AVE7688 and ramipril are manufactured by Aventis Pharma Deutschland (Frankfurt am Main, Germany) AVE7688 is a potent simultaneous inhibitor of two peptidases, ACE and neutral endopeptidase 24.11. The chemical name of AVE7688 is 7-[[(2S)-2-(acetylthio)-1-oxo-3-methylpropyl]amino]-1,2,3,4,6,7,8,12b-octahydro-6-oxo-(4S,7S,12bR)-pyrido-[2,1-a][2]benzapin-4-carboxy-acid, with a molecular weight of 432.54 (Fig. 1). In vitro, half maximal inhibitory concentrations (IC_{50}s) were determined as 0.053 nmol/l for ACE and 5.0 nmol/l for neutral endopeptidase [13]. An in vitro selectivity screen revealed no relevant binding (less than 25% at 10 μmol/l) to a large variety of receptors.

Animals. The animal experiments were carried out in accordance with the Aventis Laboratory Animal Science and Welfare (LASW) guidelines and the German law for the protection of animals. The "Principles of laboratory animal care" (NIH publication no. 85–23, revised 1985) were followed.

Male Zucker diabetic fatty rats (ZDF/Gmi-fa/fa) and their heterozygous lean littermates (ZDF/Gmi-+/fa) were purchased from Charles River Germany (Sulzfeld, Germany) and housed in the local LASW facilities in Frankfurt-Hoechst. The animals were housed individually in standard cages and received a standard chow diet (standard diet #1320, Altromin, Lage, Germany) and tap water ad libitum.

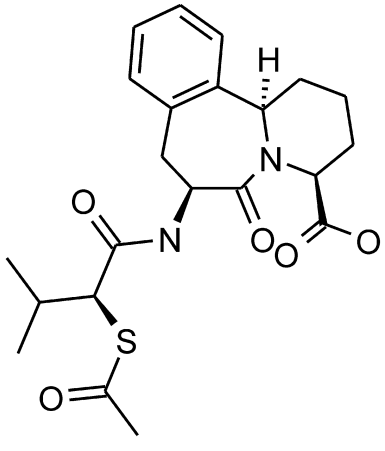

Fig. 1. Chemical structure of AVE7688

Acute pharmacodynamics. In preliminary studies, we assessed the ability of AVE7688 to inhibit serum ACE activity. Normal Wistar rats weighing 250 to 350 g were treated by single dose gavage. AVE7688 was dissolved in 1 ml hydroxy-ethyl cellulose and given to normal Wistar rats (250 to 350 g) per gavage at different concentrations ranging from 0.01 to 1.0 mg/kg (each dose group, n=4). The rats were anaesthetized 20 min later with pentobarbital (50 mg/kg i.p.) and artificially ventilated. Blood samples were taken via a polyethylene catheter from the carotid artery at 45 min, 2 h, and 4 h after administration. Thereafter, the animals were killed under ongoing anaesthesia.

Haemodynamics. The haemodynamic effects of AVE7688 were measured after chronic treatment with two representative doses. Homozygous, diabetic ZDF rats aged 36 weeks were treated with either placebo or AVE7688 at 3 or 30 mg/kg/d in chow (each group n=6). After 6 weeks, heart rate and systemic blood pressure were measured using the tail cuff method; 20 heterozygous, lean animals served as controls.

Albuminuria study. We randomly assigned 56 ZDF rats aged 34 weeks to either of five groups: the Placebo group did not receive specific treatment. The other four groups (each n=9) received AVE7688 pressed into standard Altromin chow at concentrations of 30 ppm, 100 ppm, 300 ppm, or 600 ppm. Taking into account an approximate body weight of 370 g and a daily food intake of approximately 50 g these concentrations in chow correspond to expositions of 3, 10, 30, and 60 mg AVE7688/kg/d, respectively. The 11 heterozygous, lean rats serving as non-diabetic controls (LEAN) received standard chow. All animals received Enrofloxacin (Baytril, Bayer, Leverkusen, Germany; 2.5% in drinking water) as a prophylaxis against urinary tract infections. Body weight as well as food and water intake over 24 h were determined at the beginning of the study (i.e., at age 34-weeks) and after 10 weeks of chronic therapy. At these time points, blood was taken from the tail for determination of HbA_{1c}. Simultaneously, random urine samples were collected.

Laboratory measurements. Creatinine (in urine and serum) and HbA_{1c} (in whole blood) were determined using standard kits (Roche diagnostics, Basel, Switzerland) on a Hitachi 912 E analyzer; (Hitachi, Mountain View, Calif., USA). Urinary albumin was determined using a fluorescence dye binding assay (Mikroflural, Progen Biotechnik, Heidelberg, Germany) and normalized by urinary creatinine concentration. For determination of ACE activity, the hydrolysis of the tripeptide N-[3–2-(furyl)acryloyl]-L-phenylalanyl-glycyl-glycin (FAPPG) into furylacryloyl-phenylalanine and glycine-glycine was measured spectrophotometrically as the decrease in absorption at λ= 340 nm in comparison to known standards. Serum ACE activity was always measured freshly on the day of the experiment, without intermittent freezing. All other serum and urine samples were frozen and stored at −20°C until the day of analysis when necessary.

Statistical analysis. Differences between groups were tested using unpaired Student's t-tests, corrected for multiple according to Bonferroni-Holm. Due to the clear distinction in the major outcome parameters between lean and obese animals, differences were not tested against the lean control group. Data are given as means±SEM. A p value of less than 0.05 was considered statistically significant.

Table 1. Metabolic parameters

	Lean control	ZDF obese				
		Placebo	AVE 3	AVE 10	AVE 30	AVE 60
	Body weight, g					
34 weeks	437±5	368±10	366±8	370±6	372±8	387±9
44 weeks	463±5	353±8	364±9	369±8	370±10	386±8
	Food intake, g/d					
34 weeks	21±1	47±1	46±2	48±1	48±2	49±1
44 weeks	23±0.4	48±2	49±3	48±3	51±2	51±1
	Water intake, ml/d					
34 weeks	24±2	213±7	211±10	219±9	225±10	232±8
44 weeks	43±8	214±9	221±15	233±22	235±12	244±8
	HbA1c, %					
34 weeks	4.9±0.01	12.2±0.2	12.5±0.3	12.6±0.2	11.8±0.3	12.2±0.2
44 weeks	4.9±0.02	11.5±0.2	11.3±0.3	11.2±0.3	11.7±0.2	11.5±0.6

Values are means±SEM. *n*=9 (ZDF obese groups), *n*=11 (Lean controls). AVE, chronic treatment with AVE7688 in chow. The numbers after "AVE" refer to the daily average dose (mg/kg/d)

Table 2. Urinary creatinine and albumin excretion

	Lean control	ZDF obese				
		Placebo	AVE 3	AVE 10	AVE 30	AVE 60
	Urinary albumin concentration, mg/l					
34 weeks	71±7	772±126	1175±218	953±114	891±55	1436±275
44 weeks	86±17	1321±327	1448±405	725±157	625±135	634±122 *
	Urinary creatinine concentration, mmol/l					
34 weeks	9.63±0.70	0.30±0.03	0.44±0.06	0.40±0.03	0.36±0.02	0.45±0.05
44 weeks	13.06±1.02 *	0.62±0.06 *	0.83±0.18 *	0.69±0.05 *	0.61±0.04 *	0.62±0.06 *
	Urinary albumin/creatinine ratio, mg/mg					
34 weeks	0.07±0.01	22.7±3.6	24.5±3.0	21.3±2.0	22.7±1.9	29.0±3.9
44 weeks	0.06±0.01	20.7±5.9	14.7±2.2 *	9.0±1.8 *	9.1±1.9 *	9.2±1.9 *

Values are means±SEM. *n*=9 (ZDF obese groups), *n*=11 (Lean controls). AVE, chronic treatment with AVE7688 in chow. The numbers after "AVE" refer to the daily average dose (mg/kg/d). *p<0.05 vs 34 weeks

Results

Acute pharmacodynamics. AVE7688 reduced serum ACE activity in a dose dependent manner. At 120 min after gavage, ACE activity was reduced from 160±9 U/l (placebo) to 53±4, 25±3, 22±4, 19±1, and 11±1 after 0.01, 0.03, 0.1, 0.3, and 1.0 mg/kg, respectively (all p<0.05 vs placebo). The ACE activities at the time points 45 min and 240 min were not different from the 120 min values (details not shown).

Haemodynamics study. Heart rate was 351±11, 311±8, 321±11, and 312±7 bpm in the lean, ZDF placebo, AVE 3 mg/kg/d and AVE 30 mg/kg/d groups, respectively. These values were not different from each other. Systolic blood pressure was 154±3, 156±3, 152±3, and 143±5 mmHg in the lean, ZDF placebo, AVE 3 mg/kg/d, and AVE 30 mg/kg/d groups, respectively. These values were not different from each other.

Albuminuria study. Fundamental and metabolic data at weeks 34 and 44 are given in Table 1. Obese animals had established diabetes mellitus, as manifested by excessive food and water intake and substantially increased HbA_{1c} values, compared to lean controls. Of note, AVE7688 did not influence metabolic control, as evidenced by the lack of effect on HbA_{1c} and on food intake.

Data on urinary creatinine and albumin are given in Table 2. Urinary albumin was markedly increased in

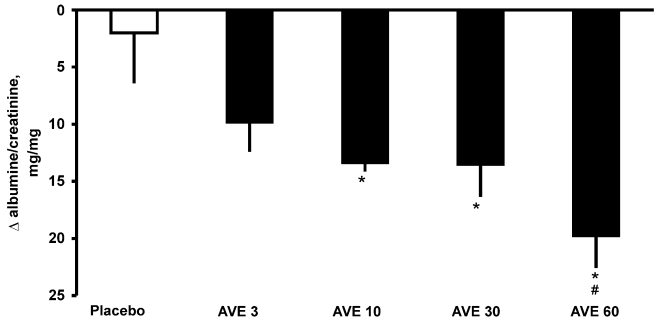

Fig. 2. Decrease in albuminuria produced by different daily doses of AVE7688 in ZDF rats. There is a dose dependent decrease in albumin/creatinine ratio by chronic treatment for 10 weeks, starting at age 34-weeks. *p<0.05 vs Placebo; # p<0.05 vs AVE 3 mg/kg/d

all obese ZDF rats, compared to lean controls, whether expressed as a crude concentration or normalized to urinary creatinine. After 10 weeks of treatment, the urinary albumin/creatinine ratio was reduced in all dose groups. This reduction was approximately 66% in the 60 mg/kg/d group and greater compared to the 3 mg/kg/d group (p<0.05). The differences in albumin/creatinine ratio between weeks 34 and 44 are illustrated in Fig. 2.

Discussion

Diabetic nephropathy and albuminuria in the ZDF rat. Diabetic nephropathy is expected to remain the leading cause of end stage renal failure in the Western world, despite the recent therapeutic advances brought about by the angiotensin receptor blockers. Albuminuria is an early and easily measurable biomarker of nephropathy [14]. The extent of albuminuria correlates with the risk of future cardiovascular events (myocardial infarction, stroke) and predicts the incidence of end stage renal failure even before retention parameters such as creatinine are increased in the serum [2, 15]. For these reasons, we used urinary albumin excretion as a validated marker of nephropathy in this study. In the ZDF (Gmi-fa/fa) rat, the development of nephropathy is characterized by progressive albuminuria and histopathological changes resembling human diabetic nephropathy [13, 16]. Interestingly, there seems to be a transient period of glomerular hyperfiltration (measured as an increased creatinine clearance at age 27-weeks) in the ZDF rat, which is very similar to the natural time course of diabetic nephropathy in humans. However, serum creatinine levels are not increased until age 37 weeks [13]. Thus, the ZDF rat model reflects a relatively early clinical stage of diabetic nephropathy.

Effect of vasopeptidase inhibition. Diabetic nephropathy is the result of a complex interplay between metabolic and haemodynamic factors [17]. Recent studies show that optimization of glycaemic and blood pressure control can reduce microvascular complications of diabetes by up to 50% [18]. Anti-hypertensive agents interrupting the renin angiotensin system have been particularly effective in retarding the progression of diabetic nephropathy [3, 4, 6]. Based on its potent and specific simultaneous inhibition of both ACE and neutral endopeptidase, AVE7688 represents a novel class of pharmacological compounds, the vasopeptidase inhibitors [9]. The decrease in serum ACE activity, observed in our study, shows that AVE7688 is a potent inhibitor of ACE also in vivo. Unfortunately, we have not been able to measure the activity of neutral endopeptidase, as there is no reliable assay available so far. However, the prototype vasopeptidase inhibitor, omapatrilat, which has similar in vitro potency as AVE7688 [9], has proven superior efficacy over pure ACE inhibitors in a variety of models [11, 19]. Future studies will show whether AVE7688, too, is superior to pure ACE inhibition and whether this is mediated through its inhibition of neutral endopeptidase.

Our data provide evidence that vasopeptidase inhibition can ameliorate diabetic nephropathy, even if treatment is started when diabetes and concomitant nephropathy are already established. The dose dependency of this effect indicates that the nephroprotection is related to the mechanism of action of the compound. The extent of the anti-proteinuric effect (up to 66% reduction) is substantial. For comparison, single therapy in advanced diabetic nephropathy has reduced proteinuria by approximately 30% in clinical trials [4, 6]. Combination therapy using an ACE inhibitor plus angiotensin receptor blocker has recently shown to decrease proteinuria in non-diabetic nephropathy by 75% [20]. It remains to be investigated whether the addition of an angiotensin receptor blocker to AVE7688 can reduce proteinuria even further.

AVE7688 did not influence glycaemic control in this study, as proven by stable HbA_{1c} values. Inhibition of ACE could be able to ameliorate insulin resistance and, consequently, reduce the incidence of diabetes mellitus. This has been shown clinically with the ACE inhibitor ramipril in the HOPE trial [21] and more recently with the vasopeptidase inhibitor omapatrilat in an experimental study in young Zucker rats [19]. It is important to note that in our study treatment was started at a relatively late stage, when diabetes was already fully established. The absence of an effect on HbA_{1c} therefore does not exclude an effect on insulin sensitivity. The potential insulin sensitizing effects of AVE7688 need to be explored in future studies, using experimental settings specifically suitable to assess insulin resistance.

In the ZDF rat, the blood pressure lowering effects of AVE7688 seem to be minimal, which is in contrast to the potent antihypertensive effects of AVE7688 [22] and other vasopeptidase inhibitors [12, 23, 24,

25] in humans and in different rat models. A possible explanation for the absence of a relevant blood pressure effect is the fact that the ZDF rat is not a hypertensive animal model. Interestingly, the angiotensin receptor blocker losartan does not protect from nephropathy in the Zucker rat despite a reduction in blood pressure [26]. The dual mode of action, particularly the effect on kinin metabolism in addition to intervention in the renin angiotensin system, could explain the dramatic efficacy of the vasopeptidase inhibitor AVE7688 in this model of Type 2 diabetic nephropathy. In a previous study, chronic treatment with an effective dose of AVE7688 (45 mg/kg/d) had no influence on creatinine clearance when the rats were older than 27 weeks [13]. We believe, therefore, that the anti-proteinuric effects of AVE7688 are not mediated via relevant decreases in the glomerular filtration rate in the ZDF rat, although we did not measure serum creatinine in the present study. Increasing evidence suggests a crucial role of the intra-renal renin-angiotensin system in diabetic nephropathy [27]. In contrast, vasopeptidase inhibition seems to afford superior protection from diabetic nephropathy compared to pure ACE inhibition [12, 13]. These observations suggest a substantial role of kinin metabolism in the pathogenesis of diabetic nephropathy. However, the relative importance of neutral endopeptidase compared with ACE for the must be clarified in future studies The vasopeptidase inhibitor AVE7688 provided effective nephroprotection in the absence of metabolic effects and major systemic blood pressure effects. It therefore seems that local renal mechanisms account for a major portion of the anti-albuminuric effects of AVE7688. If this mode of action can be confirmed in clinical studies, the vasopeptidase inhibitors will be able to support the pharmacological armentarium for the treatment of diabetic nephropathy beyond established therapeutic principles such as blood pressure normalization, glucose control, and lifestyle modifications.

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