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Original Article

The peroxisome proliferator-activated receptor-α (PPAR-α) agonist, AVE8134, attenuates the progression of heart failure and increases survival in rats

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Aim: To investigate the efficacy of the peroxisome proliferator-activated receptor-α (PPARα) agonist, AVE8134, in cellular and experimental models of cardiac dysfunction and heart failure.
Methods: In Sprague Dawley rats with permanent ligation of the left coronary artery (post-MI), AVE8134 was compared to the PPARγ agonist rosiglitazone and in a second study to the ACE inhibitor ramipril. In DOCA-salt sensitive rats, efficacy of AVE8134 on cardiac hypertrophy and fibrosis was investigated. Finally, AVE8134 was administered to old spontaneously hypertensive rats (SHR) at a non-blood pressure lowering dose with survival as endpoint. In cellular models, we studied AVE8134 on hypertrophy in rat cardiomyocytes, nitric oxide signaling in human endothelial cells (HUVEC) and LDL-uptake in human MonoMac-6 cells.
Results: In post-MI rats, AVE8134 dose-dependently improved cardiac output, myocardial contractility and relaxation and reduced lung and left ventricular weight and fibrosis. In contrast, rosiglitazone exacerbated cardiac dysfunction. Treatment at AVE8134 decreased plasma proBNP and arginine and increased plasma citrulline and urinary NOx/creatinine ratio. In DOCA rats, AVE8134 prevented development of high blood pressure, myocardial hypertrophy and cardiac fibrosis, and ameliorated endothelial dysfunction. Compound treatment increased cardiac protein expression and phosphorylation of eNOS. In old SHR, treatment with a low dose of AVE8134 improved cardiac and vascular function and increased life expectancy without lowering blood pressure. AVE8134 reduced phenylephrine-induced hypertrophy in adult rat cardiomyocytes. In HUVEC, Ser-1177-eNOS phosphorylation but not eNOS expression was increased. In monocytes, AVE8134 increased the expression of CD36 and the macrophage scavenger receptor 1, resulting in enhanced uptake of oxidized LDL.
Conclusion: The PPARα agonist AVE8134 prevents post-MI myocardial hypertrophy, fibrosis and cardiac dysfunction. AVE8134 has beneficial effects against hypertension-induced organ damages, resulting in decreased mortality. The compound exerts its protective properties by a direct effect on cardiomyocyte hypertrophy, but also indirectly via monocyte signaling and increased endothelial NO production.

Keywords: peroxisome proliferator-activated receptor; heart failure; fibrosis; inflammation; hypertrophy; nitric oxide; rat; survival

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Introduction

Peroxisome proliferator-activated receptors (PPAR) are ligand activated transcription factors which exert strong effects on metabolic pathways. Three major subtypes have been identified so far: PPARα, PPARδ, and PPARγ. All three subtypes play key roles in the regulation of lipid, carbohydrate metabolism and energy homeostasis but also in the modulation of inflammatory processes and cell differentiation[1]. PPARα, which is enriched in tissues with high oxidative energy demand, including liver, skeletal muscle, heart and kidney, stimulates the oxidation of fatty acids. It also stimulates the uptake of fatty acids and regulates the synthesis of lipoproteins. In the liver, PPARα activation enhances apolipoprotein AI (Apo AI) and Apo AII synthesis, the two major proteins of high density lipoprotein (HDL)[2]. These mechanisms constituted the basis for the clinical use of some PPARα agonists, like fenofibrate, to treat dyslipidemic patients. PPARγ activators have been approved for the treatment of type-2 diabetes mellitus, based on their efficacy in improving insulin

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sensitivity and delaying the progression of insulin resistance[3]. Despite these established pharmaco-therapies, novel PPARα, activators, which are structurally distinct and more potent than fibrates, are in clinical development for cardiometabolic abnormalities[4].

Activation of PPARα is associated with a variety of distinct signalling mechanisms and phenotypic responses. In cardiovascular cells, PPARα agonists were shown to have a protective effect on human aortic smooth muscle cells[5]. In human umbilical vein endothelial cells (HUVEC), PPARα subtype activation was linked to up-regulation of eNOS expression and reduction of glucose-induced expression of the monocyte chemoattractant protein-1 (MCP-1)[6, 7]. Furthermore, fenofibrate activates AMP-dependent kinase and increases Ser-1177 phosphorylation of eNOS in HUVEC post-transcriptionally, resulting in increased endothelial release of nitric oxide.

Apart from their established metabolic and vascular effects distinguished cardiac-specific effects of PPARα has been recently summarized[8]. Cardiac specific over-expression of PPARα increased fatty acid uptake and oxidation and decreased glucose utilization[9]. These animals developed ventricular hypertrophy and moderate systolic dysfunction. Contrariwise, mice deficient in the PPARα gene display cardiac fibrosis and lack functional cardiac response to several physiological stressors[10]. In pacing-induced heart failure in larger animals the PPARα agonist fenofibrate either prevented alterations in cardiac metabolic phenotype or slowed down the progression of left ventricular dysfunction[11, 12]. In isolated rat neonatal cardiomyocytes, both the PPARα, activators fenofibrate and WY14,643 as well as the PPARγ activator rosiglitazone were able to decrease endothelin-1 induced hypertrophy[13]. In a model of arterial hypertension (DOCA-salt model), PPARα and PPARγ activation were both able to reduce cardiac fibrosis[14]. Similar effects were also observed in a pressure overload-induced cardiac hypertrophy, induced by partial constriction of the abdominal aorta[15]. In addition, PPARα/γ activation had anti-proliferative effects, antagonized angiotensin II actions *in vivo* and *in vitro*, and exerted antioxidant actions in inhibiting generation of reactive oxygen species and activating inflammatory mediators in blood vessels and the heart[16].

In contrast to genetic gain- or loss-of-function studies, small molecules activating PPARs provide an alternative approach to modulate cardiovascular function by PPAR activation. However, only limited data are available to date for compounds with a high specificity and potency towards PPARα and their effects in models of heart failure. We therefore investigated whether activation of PPARα by the novel potent agonist AVE8134 improves myocardial function and attenuates remodelling processes in rats with permanent myocardial infarction and compared the effects to other standard treatments. In a second study, we investigated AVE8134 in DOCA-induced hypertensive rats with subsequent secondary cardiac hypertrophy and fibrosis. Finally, we explored the chronic effect of AVE8134 in old spontaneously hypertensive rats (SHR) on cardiac and renal function as well as survival. In order to identify underlying mechanisms of this new PPARα agonist, we tested AVE8134 in cellular models of hypertrophy, nitric oxide signalling and monocyte signalling.

Materials and methods

Materials

Ramipril and AVE8134, rosiglitazone and fenofibrate were synthesized in chemical departments of Sanofi-Aventis. AVE8134 (2-methyl-6-[3-(2-phenyl-oxazol-4-ylmethoxy)-propoxymethyl]-benzoic acid) is a new orally active PPARα agonist (Figure 1). It activates human and rat PPARα receptors in cellular transcription assays with nmol/L potency and is much less potent on PPARγ and PPARδ receptors (EC_{50}>3 μmol/L). The following antibodies were used for Western-blotting of lysates from tissues and cells: Polyclonal rabbit anti-phospho-eNOS (Ser1177) antibody (Zymed, San Franscico, CA, USA; final dilution 1/300); monoclonal mouse anti-eNOS (Becton Dickinson, final dilution 1/1000); monoclonal mouse anti-GAPDH (Chemicon, Germany; final dilution 1/2000).

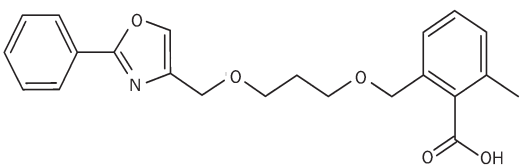

Figure 1. Chemical structure of AVE8134.

Animals

Male rats (Sprague Dawley and SHR) were purchased from møllegard, Denmark and housed in standardized conditions and diet (#1320, sodium content 0.2%, Altromin, Lage, Germany). All animal studies were performed in accordance to the German law for the protection of animals guidelines and the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996).

Myocardial infarction induced by permanent coronary ligation

Permanent occlusion of the left coronary artery close to its origin from the aorta was performed with male Sprague Dawley rats as described[18]. The day after the surgery, animals were randomized in 6 groups and treatment with AVE8134 (1, 3, and 10 $mg \cdot kg^{-1} \cdot d^{-1}$ pressed in chow) started. Within one day, therapeutic plasma levels were reached. One week after surgery, surviving rats were anesthetized and subjected to echocardiographic investigations for confirmation of successful coronary ligation. Only rats with confirmed transmural myocardial infarctions were included in the study. The mean infarct-size, as determined by planimetric analysis of removed hearts at the end of the study, was 32%±3% without significant differences between the treatment groups. After 10 weeks treatment animals were sacrificed and cardiac function was assessed in an isolated working heart preparation as described

previously[19].

Deoxycorticosterone acetate-(DOCA)-salt sensitive rats with unilateral nephrectomy

Uni-lateral nephrectomy (UNX) of the left kidney was performed on Sprague Dawley rats. After surgery, the animals were treated with DOCA (30 mg/kg sc, once weekly) and 1% sodium chloride in drinking water. The animals were randomized in three groups: (1) Controls with UNX with normal chow and normal water, (2) Placebo with UNX, DOCA-treatment and 1% NaCl in drinking water (UNX/DOCA), (3) UNX/DOCA and AVE8134 (3 $mg \cdot kg^{-1} \cdot d^{-1}$ in chow). After 4 weeks treatment systolic blood pressure was measured non-invasively via the tail cuff method and endothelial function was assessed in excised rings of the thoracic aorta as described previously[20].

Chronic treatment of SHR

Male SHR (350 to 390 g) were randomly assigned at the age of 15 months to either placebo treatment or treatment with AVE8134 (0.3 $mg \cdot kg^{-1} \cdot d^{-1}$ pressed in chow). Each group consisted of 30 animals. In pilot studies the selected dose of AVE8134 did not result in blood pressure lowering or adverse effects. After six months of treatment, approximately 70% of the placebo animals had died and an intermediate analysis of cardiac function and morphology, aorta and kidney function was performed on 9 animals per group. Treatment was then continued in the remaining animals till death.

Determination of eNOS protein expression and phosphorylation

The apex of the left ventricle was used to determine eNOS and phospho-eNOS protein content by specific Western blotting. Tissues were rapidly washed in ice-cold phosphate-buffered saline and snap frozen in liquid nitrogen. Thereafter, they were ground at the temperature of liquid nitrogen using a 6750 Spex-Freezer-mill (C3-Analysentechnik, Germany) and the powders extracted for 1 h on ice with a lysis buffer consisting of 10 mmol/L Tris-HCl, pH 7.6, 1% sodium dodecylsulfate (SDS) and protease inhibitors (Complete™, Roche). After centrifugation at 4 °C for 30 min and >100 000×*g*, supernatants were diluted with 5×Laemmli buffer and subjected to eNOS Western blotting using specific antibodies either against eNOS or Ser-1177 phosphorylated eNOS. Bound antibody was detected and quantified using infra-red-dye labelled antibodies on a Odyssee scanning device (Li-Cor, Lincoln, USA). For detection of specifically bound primary antibodies, near-infrared fluorescence labelled secondary antibodies were used (Li-Cor, Lincoln, USA, final dilution 1/15 000) and fluorscence was detected using an Odyssee imaging device (Li-Cor, Lincoln, USA). The term Ser-1177 phosphorylation is used throughout the whole manuscript for simplification, although in the rat sequence the identical phosphorylation site is indeed located at Ser-1176.

Determination of pre-clinical biomarkers

For biomarker determination, individual urine sampling was performed over 24 h in metabolic cages and retro-bulbary blood taken under light anaesthesia on the following day. In all final investigations, the hearts were weighed (myocardial hypertrophy) and a part of the left ventricle was taken to determine the ratio of hydroxyproline to proline and collagen content as described[22]. Plasma concentrations of AVE8134 were measured by HPLC. Creatinine was quantified in serum and urine with a standard kit (Roche Diagnostics) on a Hitachi 912 E analyzer. Urinary albumin was measured using a fluorescence dye binding assay (Mikroflural, Progen Biotechnik GmbH, Heidelberg, Germany).

We determined in our studies on a regular basis plasma citrulline and arginine, plasma pre-pro-BNP, plasma CRP and urinary NO(x). Determination of arginine and citrulline in rat plasma was performed by liquid chromatography – tandem mass spectrometry. The plasma concentration of pro-BNP was determined in plasma using a radio immunoassay (Phoenix Pharmaceuticals Inc.; USA). CRP was measured with an ELISA (Helica Biosystems Inc; USA). Urinary NOx was analyzed as sum of NO_3^- and NO_2^- by Capillary Electrophoresis (PA 800; Beckman) using the buffer of an "Inorganic Anion Analysis kit" (PN 5063-6511) from Agilent Technologies. Plasma triglycerides and glucose concentrations were measured using standard kits on a Hitachi 912 E analyzer.

Isolation of rat cardiomyocytes and determination of cellular hypertrophy

Adult rat ventricular cardiomyocyte cultures were obtained from hearts of 250−300 g male Sprague-Dawley rats as described[23] with minor modifications. Briefly, the hearts were excised and a retrograde perfusion was performed using as digesting enzyme Liberase-Blendenzyme IV (Roche, Mannheim, Germany). Thereafter, atria were removed, and ventricles were cut into pieces and minced. After filtering through a nylon mesh, cells were centrifuged and extracellular calcium was stepwise increased (200 μmol/L, 400 μmol/L, and 1 mmol/L) by additional 3 centrifugation steps. After isolation cardiomyocytes were suspended in serum-free medium containing 1 mmol/L $CaCl_2$. Two hours after plating, cultures were washed in medium without fetal calf serum followed by induction and measurement of hypertrophy described[24].

Nitric oxide signalling in cultivated human endothelial cells

HUVEC were isolated as described[25]. The isolation and experiments conforms with the principles outlined in the Declaration of Helsinki for the use of human tissues. Effects of AVE8134 on eNOS protein expression and phosphorylation at Ser-1177 were determined in confluent cells pre-plated in 24-wells plates, after lysis in 1×Laemmli sample buffer and specific Western blotting as described above for tissue lysates.

Cultivation and treatment of monocytes

The monocytic cell line MonoMac-6 was purchased from the German Resource Centre for Biological Material (DSMZ) with the agreement of Prof L Ziegler-Heitbrock. The cells were cultured according to the suppliers recommendations. For

experiments, cells were resuspended at a density of 2.5×10^5 cells per mL in culture medium, seeded in 12-well plates and subsequently incubated for 16 h with PPAR agonists. For uptake measurements of ox-LDL, cells were treated for 24 h with AVE8134 and then exposed to 20 μg/mL DIL-labelled oxidised LDL (Intracel, USA) for 4 h. After washing, incorporation of DIL-label was quantified by FACS analysis. Results were normalized with values obtained from control cells. Results represent average of experimental triplicates, with mean fluorescence of 50 000 cells quantified in each experiment.

RNA isolation, reverse transcription and real-time PCR

RNA was isolated using RNeasy mini kit (Qiagen) according to the suppliers protocol. The total RNA 500 ng was reverse transcribed using ImProm-II Reverse Transcription System (Promega). Real-time PCR was performed with DyNAmo Probe qPCR Kit (Finnzymes) and Assays-on-Demand (Applied Biosystems) using 5 μL of 1:10 diluted cDNA per 25 μL reaction on a Biorad i-cycler. All results were normalised with values obtained from the quantification of GAPDH expression levels and represented as percent of control samples.

Data analysis and statistics

Mean values and SEM were given. Results were pre-analyzed on normality and further characterized by Levene and Dixon's test. Depending on the outcome of these tests, parametric one way ANOVA followed if necessary by Dunnett's test, or non-parametric Kruskal-Wallis testing was employed to check significance. For all statistical analyses a P-value ≤0.05 was considered as significant. In the isolated organ experiments, a correction was made for multiple testing according to *Bonferroni-Holm*. Cumulative survival in SHR was analyzed according to Kaplan-Meier followed by Cox-mantel log rank test.

Results

Effects of AVE8134 in rats with permanent coronary ligation and subsequent cardiac re-modelling

Systolic blood pressure was significantly reduced in all rats with myocardial infarction but no differences among the different post-MI treatment groups were observed (Table 1). Body weight was normalized (comparable to sham placebo animals) by 3 mg/kg and 10 mg/kg AVE8134 treatment when compared to the post-MI placebo group. Body weight in the rosiglitazone group was increased compared to the post-MI placebo group. Weights of the lung and the left ventricle were significantly increased in the post-MI-placebo group, however, AVE8134 dose-dependently normalized these values. In contrast, lungs and hearts gained significantly more weight in the rosiglitazone group when compared to the post-MI group alone. The liver weight was dose-dependently increased in the AVE8134 groups.

AVE8134 but not rosiglitazone dose-dependently improved post-MI heart function assessed in the isolated working heart preparation (Figure 2). Indeed, AVE8134 treatment significantly improved myocardial contractility (LV dp/dt_{max}) and

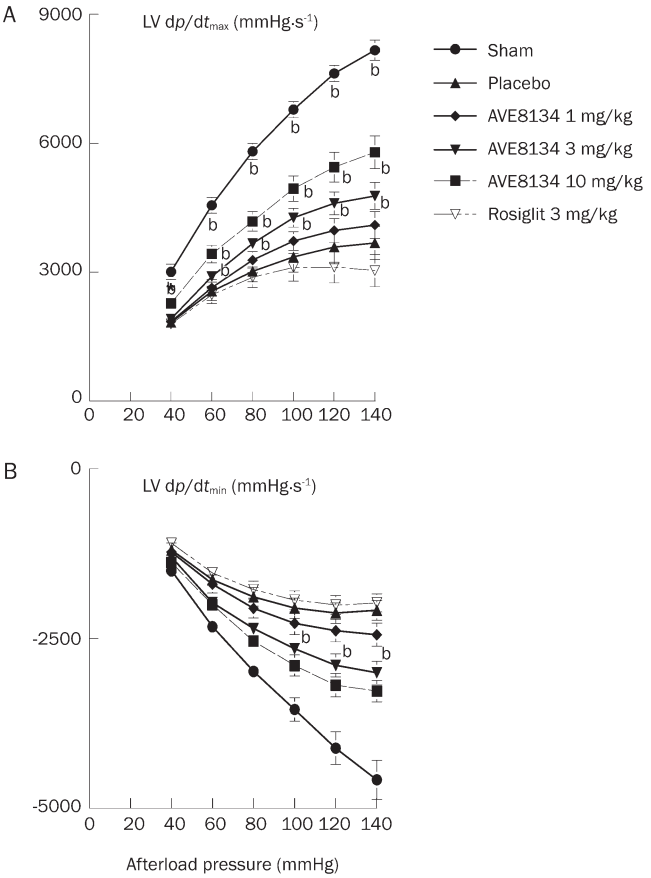

Figure 2. Effect of AVE8134 (1, 3, and 10 mg·kg^{-1}·d^{-1}) and rosiglitazone (3 mg·kg^{-1}·d^{-1}) on cardiac function post-MI. (A) Effect on left ventricular maximal contraction (dp/dt_{max}) in isolated working hearts from rats with congestive heart failure. (B) Effect on left ventricular maximal relaxation (dp/dt_{min}). Each group consisted of 12 animals. $^bP<0.05$ *vs* Placebo.

myocardial relaxation (LV dp/dt_{min}) when compared to post-MI-placebo and rosiglitazone treated rats. Cardiac output was impaired in the post-MI-placebo and rosiglitazone groups but significantly improved in the 3 mg/kg and 10 mg/kg AVE8134 groups. All these functional improvements were accompanied by decreased plasma concentrations of proBNP and arginine as well as increased plasma citrulline values and an increased urinary NOx/creatinine ratio (Table 1). Both AVE8134 and rosiglitazone decreased plasma blood glucose and serum triglycerides in the rats. The hydroxyproline/proline ratio in the heart was significantly increased in post-MI-placebo, post-MI-rosiglitazone and the lowest dosing group of AVE8134 (1 mg·kg^{-1}·d^{-1}) but was significantly reduced in the 3 and 10 mg·kg^{-1}·d^{-1} groups of AVE8134. Plasma peak expositions were achieved with the three doses of AVE8134 (1, 3, and 10 mg·kg^{-1}·d^{-1} in chow) were 0.83±0.02 μg/mL (2.2±0.05 μmol/L), 3.6±0.3 μg/mL (9.43±0.76 μmol/L) and 12.0±0.9 μg/mL, (31.4±2.4 μmol/L), respectively.

In a second post-MI rat study, AVE8134 (3 mg·kg^{-1}·d^{-1}) and ramipril (1 mg·kg^{-1}·d^{-1}) revealed similar cardioprotective effects when compared directly. Both treatments improved contractility and cardiac output, reduced lung and right ven-

Table 1. Effect of AVE8134 on cardiovascular tissues and function in rats with MI induced heart failure. [b]*P*<0.05 *vs* CHF-Placebo.

	Sham-Placebo	CHF-Placebo	CHF+AVE8134 1 mg·kg^{-1}·d^{-1}	CHF+AVE8134 3 mg·kg^{-1}·d^{-1}	CHF+AVE8134 10 mg·kg^{-1}·d^{-1}	CHF+Rosi 3 mg·kg^{-1}·d^{-1}
Blood pressure (mmHg)	138±3[b]	124±3	128±4	123±3	116±5	125±6
Body weight (g)	427±9[b]	459±8	438±7	420±8[b]	418±8[b]	485±5[b]
Lung weight (g/100 g BW)	0.39±0.01[b]	0.81±0.07	0.70±0.08	0.49±0.05[b]	0.40±0.04[b]	0.96±0.07[b]
Right ventricular weight (g/100 g BW)	0.051±0.002[b]	0.111±0.008	0.090±0.008	0.072±0.001[b]	0.063±0.006[b]	0.13±0.01[b]
Liver weight (g/100 g BW)	3.01±0.06	3.02±0.04	4.00±0.01[b]	5.07±0.08[b]	5.58±0.11[b]	2.99±0.08
Blood glucose (mmol/L)	7.45±0.33	7.62±0.23	7.23±0.22	6.89±0.18[b]	6.57±0.011[b]	6.90±0.26[b]
Serum triglycerides (mmol/L)	0.81±0.07	0.78±0.06	0.71±0.06	0.62±0.05[b]	0.41±0.03[b]	0.54±0.03[b]
Cardiac output (mL/min)	36.0±2.7[b]	7.3±1.8	12.5±2.3	21.5±2.1[b]	27.2±2.6[b]	8.7±1.6
Plasma proBNP (pg/mL)	315±8[b]	681±48	607±39	491±24[b]	387±24[b]	744±39
Plasma citrulline (μg/mL)	11.8±0.1	12.7±1.0	15.3±1.2	27.0±2.5[b]	35.1±3.2[b]	12.4±1.1
Plasma arginine (μg/mL)	15.6±1.1	16.3±1.2	16.2±1.2	11.1±1.0[b]	10.9±0.9[b]	17.5±1.4
Urinary NOx/Crea	39±4	47±5	49±5	68±6[b]	109±10[b]	26±7
Cardiac HYP/PRO ratio	0.082±0.008[b]	0.172±0.011	0.153±0.013	0.11±0.01[b]	0.090±0.009[b]	0.182±0.013

CHF=Congestive Heart Failure; NOx/Crea=nitric oxides related to creatinine; Rosi=rosiglitazone; HYP/PRO=ratio hydroxyproline/proline.

tricular weight, and reduced myocardial fibrosis, expressed as cardiac hydroxyproline/proline ratio (Table 2). Decreased plasma concentrations of pro-BNP reflected the positive effects of both treatments. In contrast to ramipril, treatment with AVE8134 clearly lowered elevated plasma CRP concentrations in post-MI animals.

Effects of AVE8134 in DOCA-salt treated rats

The combined treatment of unilateral nephrectomised rats with DOCA and sodium chloride resulted in a significant increase in blood pressure compared to non-treated groups (Table 3). This rise in blood pressure was prevented by simultaneously treating the rats with AVE8134 at a dose of 3 mg·kg^{-1}·d^{-1}. We observed no differences in body weight among the different treatment groups. However, heart weight as an overall parameter for myocardial hypertrophy and re-modeling, as well as the ratio of cardiac hydroxyproline/proline as a measure of myocardial fibrosis, were both significantly increased in DOCA/salt-treated rats. AVE8134 normalized both parameters to values observed in the placebo group without DOCA/salt treatment. Lung weight was increased in the DOCA/salt-placebo rats and was normalized again in DOCA/salt-AVE8134 treated rats. Plasma pro-BNP was significantly increased in DOCA-Placebo rats and was reduced by AVE8134 treatment. Treatment of unilateral nephrectomised rats with DOCA and NaCl resulted in a non-significant decreased cardiac protein expression and Ser-1177 phosphorylation of eNOS (Figure 3). Co-treatment with AVE8134 increased both, total eNOS protein and phospho-Ser-1177 eNOS in the hearts.

Thoracic aortic preparations responded to phenylephrine (1×10^{-7} mol/L) as follows (from baseline): control placebo 24%±2%, DOCA-placebo 74%±6% (*P*<0.05 versus placebo and AVE8134), AVE8134 36%±4%. Aortic endothelium-dependent relaxation was impaired in DOCA-placebo rats and significantly improved by AVE8134 treatment (Figure 4). The NO synthase blocker *L*-NAME totally blocked endothelium-dependent relaxation. Endothelium-independent relaxation did not reveal any differences between the groups (data not shown). The amount of total eNOS protein and phospho-Ser1177 eNOS protein in aortic lysates was non-significantly elevated by AVE8134-treatment (data not shown). Kidney

Table 2. Effects of AVE8134 and ramipril in rats with MI induced heart failure. [b]*P*<0.05 *vs* CHF-Placebo.

	Sham-Placebo	CHF-Placebo	CHF+AVE8134 3 mg·kg^{-1}·d^{-1}	CHF+Ramipril 1 mg·kg^{-1}·d^{-1}
Systolic blood pressure (mmHg)	140±6[b]	128±5	126±7	125±5
Body weight (g)	422±5[b]	453±9	427±7[b]	416±5[b]
Lung weight (g/100 g BW)	0.38±0.01[b]	0.83±0.07	0.46±0.05[b]	0.41±0.05[b]
Right ventricular weight (g/100 g BW)	0.053±0.003[b]	0.121±0.007	0.067±0.005[b]	0.061±0.007[b]
Cardiac output (mL/min)	33.3±2.9[b]	5.8±0.9	18.2±2.8[b]	19.0±2.6[b]
Cardiac HYP/PRO ratio	0.081±0.009[b]	0.162±0.012	0.103±0.008[b]	0.112±0.009[b]
Plasma proBNP (pg/mL)	324±9[b]	645±57	501±27[b]	513±32[b]
Plama CRP (μg/mL)	257±15[b]	293±10	211±12[b]	322±20

CHF=Congestive Heart Failure; NOx/Crea=nitric oxides related to creatinine; HYP/PRO=ratio hydroxyproline/proline.

Table 3. Effect of AVE8134 on systolic blood pressure, body weight, heart weight and the ratio of cardiac HYP/PRO (hydroxyproline/proline) in DOCA salt rats with UNX. [b]P<0.05 *vs* DOCA-Placebo.

	Control-Placebo	DOCA-Placebo	DOCA+AVE8134 3 mg·kg^{-1}·d^{-1}
Systolic blood pressure (mmHg)	132±2[b]	183±5	135±3[b]
Body weight (g)	413±8	377±9	391±9
Heart weight (g/100 g BW)	0.328±0.002[b]	0.422±0.082	0.341±0.025[b]
Lung weight (g/100 g BW)	0.41±0.01[b]	0.52±0.01	0.40±0.02[b]
Plasma proBNP (pg/mL)	314±12[b]	639±12	438±11[b]
Cardiac HYP/PRO ratio	0.081±0.006[b]	0.104±0.007	0.082±0.005[b]
Urinary albumine to creatine ratio	0.12±0.01[b]	1.05±0.19	0.62±0.17[b]

DOCA=deoxycorticosterone-acetate.

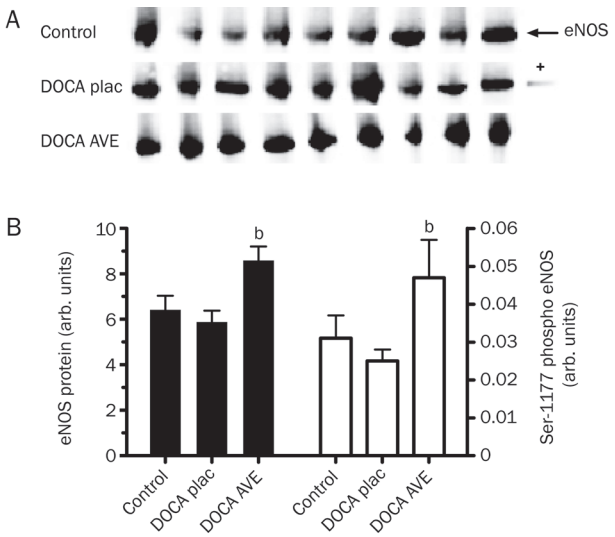

Figure 3. Myocardial expression and Ser-1177 phosphorylation of eNOS in DOCA/UNX rats treated with either placebo or AVE8134 (3 mg·kg^{-1}·d^{-1}) and compared to age-matched sham animals without DOCA-salt treatment and UNX. (A) Original eNOS protein Western blot data obtained with total cardiac lysates. After lysis, clearance and denaturation of the samples, exactly similar amounts of total protein were subjected to denaturating SDS gel electrophoresis and Western blot. A HUVEC lysate (+) was used as control. (B) Overall quantification of the samples analyzed in A. Shown are mean western blot fluorescence intensity±SEM of groups; [b]*P*<0.05 *vs* DOCA-placebo treatment.

function as measured via the urine albumin/creatinine ratio was impaired in DOCA-placebo rats and clearly improved in DOCA-AVE8134 treated animals (Table 3).

Effects of chronic treatment on SHR

After chronic treatment of SHR for five months with a low dose of AVE8134 (0.3 mg·kg^{-1}·d^{-1}), blood pressure was not different compared to the placebo treated animals (Table 4). One month later (after six months) only 9 animals in the placebo group had survived. An interim investigation was performed by means of the isolated working heart preparation. Hearts from placebo treated rats showed a reduced contractility

Table 4. Effect of AVE8134 on old spontaneously hypertensive rats with congestive heart failure. *n*=9. [b]*P*<0.05 *vs* Placebo.

	Placebo	AVE8134 3 mg·kg^{-1}·d^{-1}
Systolic BP (mmHg)	206±5	205±5
Body weight (g)	358±13	354±15
Total Heart weight (g/100 g BW)	0.63±0.02	0.56±0.01[b]
Left-ventricular weight (g/100 g BW)	0.45±0.02	0.40±0.01[b]
Right-ventricular weight (g/100 g BW)	0.098±0.003	0.065±0.005[b]
Cardiac output (mL/min)	10.2±2.0	16.6±1.7[b]
Heart power (mJ·min^{-1}·g)	277.3±23.3	377.7±19.6[b]
dp/dt_{max} (mmHg·s^{-1})	5922±478	7653±302[b]
dp/dt_{min} (mmHg·s^{-1})	-2899±220	-3643±209[b]
Plasma proBNP (pg/mL)	596±73	386±41[b]
Urinary ratio of NOx to creatinine	57±6	179±21
Urinary ratio of albumine to creatinine	10.2±0.9	6.4±1.1[b]
Cardiac HYP/PRO ratio	0.22±0.02	0.13±0.01[b]

dp/dt_{max}=LV maximal first derivative of pressure development over time; dp/dt_{min}=LV minimal first derivative of pressure development over time; HYP/PRO=ratio hydroxyproline/proline.

which resulted in reduced heart function and cardiac output compared to hearts from old SHR treated with AVE8134. Myocardial hypertrophy and fibrosis, measured as ratio of hydroxyproline to proline, was significantly reduced by AVE8134 treatment. Furthermore, administration of AVE8134 significantly reduced total heart and left and right ventricle weights (Table 4), decreased plasma proBNP concentrations and increased ratios of urine NO_x to creatinine. Impaired endothelial function was improved by AVE8134 treatment by 20%. Urinary albumin excretion was also reduced by AVE8134 treatment. No differences were observed regarding phenylephrine-induced vascular contraction among the different groups (placebo 26%±3%, AVE8134 27%±2% from baseline). Most importantly, AVE8134 extended the life span of old SHR from 22 to 26 months (*P*<0.05 *vs* placebo; Figure 5).

Cellular effects of AVE8134

Real-time PCR analysis confirmed the expression of PPARα

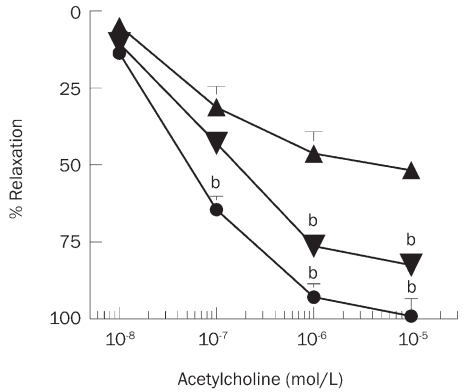

Figure 4. Endothelial function in DOCA/UNX salt-treated animals either on placebo or AVE8134 administration (3 mg·kg^{-1}·d^{-1}). Aortic rings were pre-contracted as described and endothelium dependent relaxation induced by increasing concentrations of acetylcholine. Shown are means±SEM of 10 animals per group; $^{b}P<0.05$ *vs* DOCA-Placebo. ● Control animals without DOCA/UNX salt treatment; ▲ DOCA/UNX salt placebo treated animals, ▼DOCA/UNX salt animals treated additionally with AVE8134.

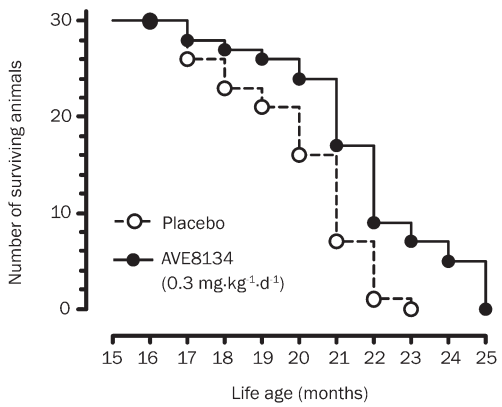

Figure 5. Survival of old SHR which were treated with either treated placebo (●) or AVE8134 at a dose of 0.3 mg·kg^{-1}·d^{-1} (o) starting at the age of 15-month.

in adult rat cardiomyocytes, HUVEC, and Monomac-6 cells (data not shown). We assessed in adult rat cardiomyocytes two markers for hypertrophy on the effect of PPAR agonists. Co-incubation of AVE8134 (1 μmol/L) for 24 h reduced phenylephrine-induced hypertrophy in both markers, cell surface area and *de novo* protein synthesis, respectively (Figure 6A and 6B). In contrast, the PPAR-γ agonist rosiglitazone reduced phenylephrine induced *de novo* protein synthesis but did not decrease cardiomyocyte surface area.

We furthermore investigated the modulation of eNOS protein expression and phosphorylation in human endothelial cells (HUVEC, Figure 7B, 7D). After incubation for 18 h, eNOS protein expression was not increased by AVE8134 (1 μmol/L). Higher concentrations where also ineffective, whereas the HMG-CoA-reductase inhibitor simvastatin (5 μmol/L) nearly doubled eNOS protein (data not shown). Rosiglitazone decreased eNOS protein expression. Both PPAR agonist show a similar trend in increasing transient phosphorylation of eNOS at Ser-1177. After a brief incubation for 5 min phospho eNOS content was doubled by AVE8134 and rosiglitazone (Figure 7A, 7C). After 60 min no effects could be observed any longer (data not shown).

In the human monocyte cell line, Monomac-6, AVE8134 concentration-dependently increased CD36 and MSR1 expression (Figure 8A). Here we observed similar effects for other PPAR agonists, specifically rosiglitazone and fenofibrate. In order to determine difference in cell function upon this increase in cholesterol transporters we measured uptake of fluorescent-labelled oxLDL and observed a significantly enhanced increase upon AVE8134 treatment (Figure 8B).

Discussion

PPAR transcription factors form important regulatory pathways in cardiac physiology and disease. Initially, interest focused on the effects of the major transcription factor subtypes on metabolic pathways. Subsequently, additional aspects, *eg* anti-inflammatory mechanisms, were identified. Despite a plethora of literature, only few studies with PPAR agonists in established heart failure models have been published so far. In humans, heart failure covers a range of disease syndromes with still increasing prevalence. Even under optimal pharmacotherapy, mortality remains high in heart failure patients, and new approaches are needed. We therefore were interested to test the effects of the novel, potent and selective PPARα agonist AVE8134 in pre-clinical models of cardiac re-modelling and failure.

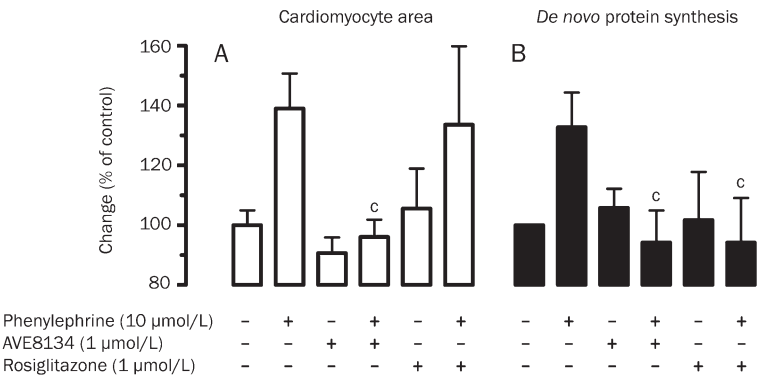

Figure 6. Effects of PPAR agonists on phenylephrine induced hypertrophy of adult rat cardiomyocytes. (A) Cells were co-incubated with the α-adrenergic stimulus phenylephrine (10 μmol/L) and PPAR agonists at 1 μmol/L and hypertrophy measured as increases in cell surface area. Shown are means±SEM from 3 independent cell preparations. $^{c}P<0.01$ *vs* phenylephrine treatment. (B) Effects on *de novo* protein synthesis.

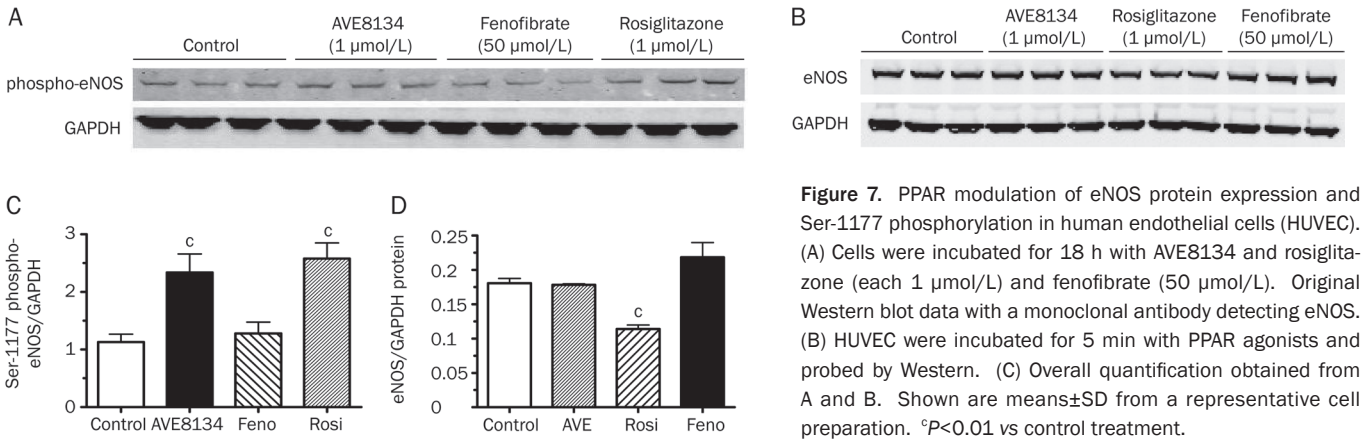

Figure 7. PPAR modulation of eNOS protein expression and Ser-1177 phosphorylation in human endothelial cells (HUVEC). (A) Cells were incubated for 18 h with AVE8134 and rosiglitazone (each 1 μmol/L) and fenofibrate (50 μmol/L). Original Western blot data with a monoclonal antibody detecting eNOS. (B) HUVEC were incubated for 5 min with PPAR agonists and probed by Western. (C) Overall quantification obtained from A and B. Shown are means±SD from a representative cell preparation. $^{c}P<0.01$ *vs* control treatment.

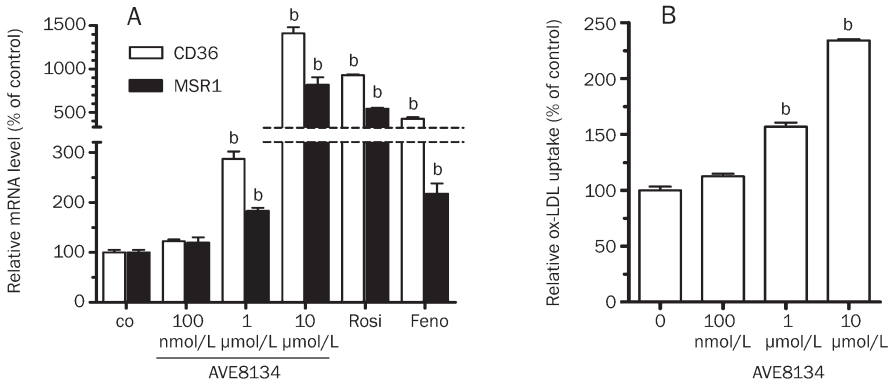

Figure 8. Effects of AVE8134, rosiglitazone and fenofibrate on scavenger receptors in cultured human monocytes. (A) Human MonoMac-6 cells were incubated respectively for 18 h. Thereafter mRNA expression was measured for CD36 and MSR1 by realtime-quantitative PCR. (B) Uptake of fluorescent labelled ox-LDL into monocytes. Cells were pre-treated for 24 h with the respective treatments, before fluorescent ox-LDL was added for further 4 h incubation. n=3. Mean±SEM. $^{b}P<0.05$ *vs* control treatment.

Ischemic heart failure induced by permanent coronary ligation

In our post-MI studies with permanent occlusion of the left coronary artery in rats, cardiac function clearly deteriorated within 8 weeks after surgical intervention. Long-term treatment with AVE8134 dose-dependently improved cardiac function.

At a dose of 3 $mg \cdot kg^{-1} \cdot d^{-1}$, the PPARα agonist showed similar efficacy to the potent ACE-inhibitor ramipril, thus demonstrating the potential of this novel approach. Moreover, right ventricular weight, body and lung weight, all indicators for congestion, were even corrected with higher doses of AVE8134 to values approaching those of sham operated animals.

Several biomarkers paralleled the functional improvements achieved with AVE8134 in this model. Anti-fibrotic effects could be demonstrated by measuring the ratio of cardiac hydroxyproline to proline, which increased in the heart failure animals and was normalized by administration of the PPARα agonist. Compound administration resulted in decreased plasma proBNP concentration, which is a validated plasma biomarker for heart failure in human patients[26]. In addition, AVE8134 increased in this model the ratio of urinary NO_x to creatinine. NO_x is the sum of nitrite and nitrate, both formed as stable end products of biologically labile nitric oxide. The urinary concentration of NO_x is linearly related to its circulating level[27]. Rather surprisingly, we noticed increased plasma citrulline and decreased plasma arginine concentrations in animals treated with AVE8134. All these nitric oxide biomarker changes support the hypothesis, that treatment with AVE8134 may increase the activity of nitric oxide synthases in the circulation, with increased citrulline concentrations as a bi-product of increased NO release from arginine.

Beside improvement of vascular nitric oxide signaling, anti-inflammatory components may contribute in an important fashion to the mode-of-action of AVE8134. In the rat post-MI model we observed a reduction in plasma concentrations of C-reactive protein (CRP) by AVE8134. CRP is an acute phase protein, whose plasma levels increase strongly during inflammatory processes. It is thought to assist in complement binding to foreign and damaged cells and affects thereby the humoral response to disease. Recent research suggests that patients with elevated basal levels of CRP are at an increased risk for diabetes, hypertension and cardiovascular disease[28,29]. In patients with chronic post-MI, the elevated levels of CRP observed were suggested to have an independent prognostic value for this disease[30].

The effects of PPAR-γ activation in pre-clinical heart failure models induced by coronary ligation remain controversial. The highly potent pioglitazone improved left ventricular remodelling in mice hearts subjected to myocardial infarction[17]. Frantz and co-workers challenged these results when demonstrating no maladaptive but also no beneficial effects in a similar mouse model[31]. As one

major methodological difference they started treatment not immediately after myocardial infarction but with a delay of 7 d, which may reflect more a clinical situation. In rats with post-myocardial infarction, treatment with rosiglitazone did not alter remodelling but increased mortality[32]. We obtained different results in our post-MI model with aggravated cardiac dysfunction. In accordance with Lygate and co-workers[32], we started the rosiglitazone treatment early after surgery with a dose of 3 $mg \cdot kg^{-1} \cdot d^{-1}$ mixed in chow for 8 weeks. However, the mean infarct size (32%±3%) was much lower in our study. This difference may well explain the differences observed in cardiac function. A common conclusion may be that at least protective effects of PPAR-γ agonism in left ventricular remodelling cannot be uniformly demonstrated in pre-clinical models. Recently a meta-analysis of several studies has been published implying an increased risk of myocardial infarction and death from cardiovascular causes by rosiglitazone[33]. In 2008, the FDA added "black box" warnings about increased heart failure risk with rosiglitazone and pioglitazone[34, 35].

Efficacy of AVE8134 on heart failure as a consequence of renal deterioration and subsequent hypertension

In order to confirm a role of the PPARα agonist AVE8134 in additional pre-clinical heart failure patho-physiologies, we utilized an animal model in which cardiac damage was caused not by myocardial infarction but rather by hypertension. By using DOCA and salt treatment in unilateral nephrectomized rats, we generated hypertension with subsequent myocardial hypertrophy and fibrosis, and endothelial dysfunction. Treatment with AVE8134 decreased systolic blood pressure to a level similar to sham-treated animals. Furthermore, reduced myocardial hypertrophy and fibrosis, and improved endothelial function were observed.

Moreover, pro-BNP was significantly reduced and the increased lung weight (indicator of congestion) was normalized in the AVE8134-treated group. Interestingly, impaired kidney function was also significantly improved by AVE8134 treatment. A potential mechanism by which AVE8134 exerts its beneficial effects is restoration of the eNOS/NO signalling pathway. In our study we observed a decrease in eNOS expression in animals treated with DOCA/salt alone, which could be normalized to sham values by treatment with AVE8134. Furthermore, the content of cardiac Ser-1177-phosphorylated eNOS, a surrogate for active enzyme, was increased by the PPARα agonist. Similar results were found by Newaz and co-workers with clofibrate exerting beneficial cardiovascular effects via increased NO production and/or inhibition of NAD(P)H oxidase activity[36]. Moreover, there is evidence that PPARα activation leads to improved nitric oxide availability by inhibition of the formation of reactive oxygen species [6,16].

Chronic treatment of SHR

The old SHR is an animal model of hypertension-induced heart failure and end-organ damage. SHR die from cardiovascular complications at an age of about 15–22 months after a long period of stable compensated cardiac hypertrophy as a result of the persistent hypertension. The cardiovascular complications mainly involve ventricular fibrillation, resulting from multiple micro-infarctions, and heart failure. In such old SHR, chronic low dose treatment with AVE8134 (0.3 $mg \cdot kg^{-1} \cdot d^{-1}$) had no effect on blood pressure but reduced myocardial hypertrophy, improved cardiac and vascular function as well as kidney function. As in other heart failure models, plasma proBNP was significantly reduced and urinary NOx/creatinine ratio increased. All these effects converged finally in a significant increase in the life expectancy of AVE8134-treated animals. These beneficial effects with the PPARα agonist AVE8134 were similar to those previously observed in SHR treated with an ACE-inhibitor, AT-1 blocker or a vasopeptidase inhibitor[20]. However, the beneficial effects of AVE8134 are clearly blood pressure independent and probably also due to a combination of different mechanisms.

Comparison of AVE8134 to other PPARα agonists

Fenofibrate was shown to prevent the development of hypertension, and decreased myocardial inflammation and collagen deposition in Ang II-infused rats[37]. In rats with aortic banding, fenofibrate treatment inhibited left ventricular hypertrophy and phenotypic changes in cardiac gene expression of endothelin-1, BNP, and β-myosin heavy chain[38]. In a model of chronic pressure overload model (transverse aortic constriction) fenofibrate showed adverse effects in PPARα-deficient mice, such as activation of MMP, increased fibrosis, left ventricular hypertrophy and increased mortality[39]. In PPARα wild type mice, fenofibrate treatment showed opposite (beneficial) effects. These observations underline the important role of PPARα activation in pressure overload-induced cardiac remodelling. In pacing induced heart failure in chronically instrumented dogs, fenofibrate prevented changes in myocardial substrate metabolism (down-regulation of the free fatty acid oxidative pathway and marked increase in glucose oxidation). This was accompanied by a modest improvement of cardiac function during the progression of the disease with no effects on the onset of decompensation[11]. In pacing induced heart failure in pigs, treatment with fenofibrate slowed down the progression of left ventricular dysfunction[12].

The dose-dependent increase in liver weight in the AVE8134 groups is most probably PPARα-related. The hepatomegaly is a rodent-specific effect of fibrates and other PPARα agonists like AVE8134. Exposure of rodents to PPARα initiates short-term pleiotropic responses including hepatomegaly, peroxisome proliferation, and increases fatty acid oxidation in liver, kidney, and heart through induction of genes encoding enzymes for fatty acid metabolism[40].

Most of the beneficial results published with PPARα agonist in models of cardiac dysfunction seems to differ strikingly to effects described by cardiac specific genetic over-expression of PPARα[9]. These transgenic mice display increased fatty acid uptake and oxidation, decreased glucose utilization and develop ventricular hypertrophy and moderate systolic

dysfunction. At least in rodents, peripheral hepatic actions of PPARα agonists, may explain in part cardiac effects of the systemically administered compounds[8]. We observed in rats with coronary ligation rather strong decreases in serum triglycerides upon treatment with AVE8134 (Table 1) which may influence indirectly the metabolic status in the hearts independent of more direct PPARα-induced cardiac effects. Mice with transgenic cardiac-specific overexpression obviously lack such peripheral effects. In addition, genetic overexpression of PPARα was obtained under the control of myosin heavy chain promoter which directs all (strong) overexpression into a single cardiac cell type, the cardiomyocytes, neglecting beneficial effects on other cell types like the coronary endothelium.

A controversial point still remains in that PPARα activation may induce in the heart a shift from glucose oxidation towards more oxygen consuming fatty acid oxidation by transcriptional control of malonyl-CoA decarboxylase[41, 42]. Hence an apparent discrepancy between the putative maladaptive upregulation of cardiac fatty acid oxidation and beneficial effects of PPARα agonists on cardiac function exists. One likely explanation is, that anti-inflammatory and other mechanisms overrule in longterm-treatments changes in the metabolic status. Ichihara and coworkers demonstrated that fenofibrate prevented reductions in cardiac oxidized glutathione and upregulations of redox-sensitive transcription factors and adhesion molecules in Dahl-salt sensitive rats[43].

Direct effects of AVE8134 in cardiomyocyte and endothelial cell cultures

In order to outline underlying signalling mechanisms of AVE8134, we employed a set of cell culture models. In adult rat cardiomyocytes, AVE8134, at a concentration activating only PPAR-α, but not PPAR-γ or PPAR-δ receptors (1 μmol/L), reduced cell hypertrophy assessed by cardiomyocyte surface and *de novo* protein biosynthesis induced by the α-adrenergic stimulus phenylephrine. Rosiglitazone prevented in this setup *de novo* protein synthesis but not increases in cell surface area and hence has not a similar anti-hypertrophic effect in these cells compared to AVE8134. Fenofibrate displayed similar effects like AVE8134 (data not shown). The anti-hypertrophic effect of AVE8134 fits well to our *in vivo* data with ischemic and non-ischemic heart failure models, in which we also observed anti-hypertrophic effects on the long-term.

In primary human endothelial cells, we observed no effect of AVE8134 on eNOS protein expression. However, phosphorylation of eNOS at Ser-1177, a valuable marker for active eNOS enzyme, was transiently increased by the compound. Similar observations have been described with fenofibrate[44]. We could not confirm the fenofibrate results with our cellular setup. However, we overall used a lower final concentration (50 μmol/L), because this compound tended to precipitate at intially planned higher concentrations. Rosiglitazone displayed a similar short-term effect on eNOS phosphorylation but decreased the long-term eNOS protein expression and hence did not display a similar profile like AVE8134. In our ischemic heart failure model, AVE8134 displayed significant effects on biomarkers of the nitric oxide pathway in elevating plasma citrulline and urinary NO_x secreation and deprivation of plasma arginine. Although our cellular data fits well to the *in vivo* observation, it remains to be determined how AVE8134 increases eNOS phosphorylation. In accordance with other PPAR agonists, activation of the AMP-kinase may act upstream of eNOS.

Gene expression studies in cultured human monocytes

Beside these established effects of PPARα activation on cardiomyocyte and endothelial signal transduction, we identified a third mechanism of action of AVE8134 in monocytes which might well explain part of its efficacy under conditions of heart failure. For these experiments we adopted as a model the MonoMac-6 cell line[45, 46]. At a concentration of AVE8134 specifically activating PPARα but not the other subtypes (1 μmol/L), we observed increases in the expression of the scavenger receptor genes CD36 and MSR1. At 10 μmol/L, the effects of AVE8134 were significantly stronger compared to those of the reference PPARα-agonist fenofibrate or the PPARγ-agonist rosiglitazone. The functional consequences of the observed changes in gene expression could be confirmed since treatment with AVE8134 resulted in a concentration dependent increase in uptake of DIL-labelled ox-LDL. The scavenger receptors CD36 and MSR1 have been identified as important risk factors for foam cell- and atherosclerotic lesion formation, since they play important roles in the uptake of oxidized low-density lipoprotein (ox-LDL) by macrophages. However, several recent studies have launched a debate as to whether or not these receptors play indeed a critical role for the development of atherosclerosis[47–49]. We suggest that increased uptake of oxidized LDL into monocytes may serve as an important clearance mechanism of modified LDL's from vessel walls, thereby improving indirectly endothelial and cardiomyocyte function. Although this mechanism seemed to be mediated more potently by PPARα than PPARγ, simultaneous agonism on both subtypes may be needed to achieve its full activation. PPAR isoforms are endowed with the dual capacity to modulate both metabolism and inflammation. In this way these nuclear receptors are likely to interfere with cardiac remodelling, ultimately affecting cardiac energy homeostasis, apoptosis, fibrosis, and possibly angiogenesis[50].

Conclusion

We demonstrate that the PPARα agonist AVE8134 exerts beneficial effects in three different pre-clinical settings of cardiac dysfunction and heart failure. AVE8134 not only prevented typical re-modelling processes after myocardial infarction, but also decreased patho-physiological alterations after acute and long-standing (untreated) hypertension. Our data suggest that AVE8134 exerts its effects via a plethora of cellular signalling pathways in cardiomyocytes, endothelial cells but also monocytes. The PPARα agonist AVE8134 is not only a promising candidate for treatment of cardio-metabolic disorders but also provides an innovative approach to the treatment of heart

failure.

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Author contribution

Wolfgang LINZ and Paulus WOHLFART designed and performed and analysed most of the studies and wrote the paper. Manuel BAADER performed the research and analysis of experiments with Monomac-6 cells. Kristin BREITSCHOPF performed research and analysis of experiments with cardiomyocytes. Martin GERL designed, performed and analysed biomarker measurements in the *in vivo* studies. Eugen FALK and Hans-Ludwig SCHÄFER contributed new reagents and analytical tools. Werner KRAMER and Hartmut Rütten designed research.

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