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Leoligin, the major lignan from Edelweiss, inhibits 3-hydroxy-3-methyl-glutaryl-CoA reductase and reduces cholesterol levels in ApoE —/— mice



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ABSTRACT

The health benefit through the control of lipid levels in hyperlipidaemic individuals is evident from a large number of studies. The pharmacological options to achieve this goal shall be as specific and personalized as the reasons for and co-factors of hyperlipidaemia. It was the goal of this study to reveal the impact of leoligin on cholesterol levels and to define its mechanism of action.

Oral application of leoligin in ApoE -/- mice led to significantly reduced total serum cholesterol levels and a reduction in postprandial blood glucose peak levels. In the absence of biochemical signs of toxicity, leoligin treatment resulted in reduced weight gain in mice. The effects of leoligin on serum cholesterol levels may be due to a direct inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) by a unique, non-statin-like binding mode. Postprandial serum glucose peaks may be reduced by a mild peroxisome proliferator-activated receptor-gamma (PPAR- γ) agonistic activity of leoligin. No effect on atherosclerotic plaque size was observed.

As a non-toxic, cholesterol-, peak glucose-, and weight gain-lowering compound, leoligin continues to fulfil characteristics of a potential agent for the treatment of cardiovascular disease (CVD). The counterregulatory overexpression of hepatic HMGCR in leoligin treated animals possibly explains the missing permanent antiatherosclerotic effect.

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1. Introduction

In 1913, Anitschkow and Chalatov showed that a cholesterol rich diet in rabbits produces vascular lesions similar to atherosclerotic lesion in humans [1]. Consequently, numerous animal, clinical, and epidemio-logical studies elucidated the association and causality between dyslipidaemia, the development of atherosclerosis, and coronary artery disease (CAD) mortality. Important pioneering work which cleared the way for the lipid-hypothesis of atherosclerosis included i) the identification of HMGCR as the rate-limiting enzyme of cholesterol biosynthesis [2], ii) the discovery of the low-density lipoprotein (LDL) – receptor

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[3], iii) the Framingham Heart study providing evidence for the association between hypercholesterolemia and CAD [4,5], iv) the introduction of HMGCR inhibitors, better known as statins [6], and v) the outcome of the Coronary Primary Prevention Trail in 1984, showing that a reduction of total plasma- and LDL-cholesterol results in a lower incidence of CAD [7,8]. Treatment options for atherosclerosis and CAD which include conservative approaches, such as physical exercise, diet, and pharmacotherapy [9] as well as interventional options, namely coronary artery bypass grafting and percutaneous coronary intervention are versatile and mainly depended on an individual's genetic background, life style, and the stage of the disease. In the last two decades LDL-cholesterol lowering statins have been shown to reduce mortality rates in primary and secondary prevention of CAD [10,11]. Moreover, several studies investigated the effects of statins on atherosclerotic plaque progression and demonstrated that an aggressive statin regiment results in a

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regression of coronary atherosclerosis in humans [12–14]. Despite their indispensable clinical importance and the fact that the discovery of statins and their widespread use is a tremendous success story, the search for new lipid lowering drugs is still a highly relevant task [9].

Leoligin, a compound isolated from the roots of Leontopodium nivale subsp. alpinum Cass. (Edelweiss), has shown promising anti-atherosclerotic characteristics in previous studies: Duwensee et al. investigated the influence of leoligin on cholesteryl ester transfer protein (CETP), an enzyme that promotes the transfer of cholesteryl esters from high-density lipoprotein (HDL) to, among other lipoproteins, LDL. Thus CETP inhibition raises HDL-cholesterol levels [15]. In the same study, leoligin was also shown to inhibit CETP in human plasma. Further, transgenic CETP mice given leoligin orally showed a trend towards reduced serum LDL-cholesterol levels even though elevated CETP activity was measured, suggesting a possible lipid lowering effect due to other mechanisms [16]. Additionally, the effects of leoligin on venous grafts in vitro as well as in vivo were explored. Leoligin significantly inhibited intimal hyperplasia in human saphenous veins in vitro and neointima formation in an *in vivo* venous bypass graft mouse model, without causing endothelial damage. Treatment of primary vascular smooth muscle cells and endothelial cells with leoligin revealed a significant inhibitory effect on smooth muscle cell proliferation by inducing cell cycle arrest in the G1-phase and an inhibition of tumor necrosis factor-alpha (TNF- α)-mediated endothelial vascular cell adhesion molecule-1 (VCAM-1) expression [17].

As mentioned above, leoligin was capable of reducing serum LDLcholesterol levels to a point which could not be explained by previous findings. Accordingly, the basis of the present study is formed by the hypothesis that leoligin reduces LDL-cholesterol levels in ApoE -/- mice independent from its effects on CETP activity and shall thereby prevent atherosclerotic plaque formation.

2. Material and methods

2.1. Leoligin

The isolation of leoligin from Edelweiss (*Leontopodium nivale* subsp. *alpinum*) and its purification (98% purity) has been described previously [18].

2.2. Study design

Female 24 weeks-old ApoE -/- mice on C57BL/6J genetic background were obtained from Charles River (Sulzfeld, Germany) and housed at 24 °C and a 12 h light/dark cycle in a virus/antibody-free environment of the Core Unit for Biomedical Research at the Medical University of Vienna. All animal experiments corresponded to the European Union directive 2010/63/EU, were approved by Austrian veterinary authorities, and were carried out in accordance to the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals", prepared by the Institute of Laboratory Animal Resource and published by the NIH.

ApoE -/- mice were fed a standard chow diet V1534–300 (ssniff, Germany) and received drinking water *ad libitum*. After a short period of acclimatization, ApoE -/- mice (n = 40; age = 29.5 \pm 2 weeks) were randomly divided into four treatment groups (n = 10 per group) to receive either: vehicle control (0.1% dimethylsulfoxide; Sigma-Aldrich, USA), 1 μ M leoligin, 10 μ M leoligin, or 50 μ M leoligin dissolved in drinking water (all in 0.1% dimethylsulfoxide end concentration), respectively. Anaesthesia was carried out using 7.2 mg xylasol + 96 mg ketamine (Bayer, Germany; Richter Pharma, Austria) per kg body weight.

After 5 and 10 weeks of treatment fasting blood samples for biochemical analyses were collected from the tail vein using a Microvette CB300Z (Sarstedt, Germany). After 2 and 16 weeks of treatment, intraperitoneal glucose tolerance tests (IPGTT) were performed in fasting mice (n = 5 per treatment group).

After 16 weeks of treatment, mice were anaesthetized (240 mg xylasol + 1200 mg ketamine/kg body weight) and final blood samples were obtained by puncture of the inferior vena cava, mice were sacrificed by removal of the heart and consequent terminal bleeding. Finally the aorta was extracted and carefully cleaned of all perivascular tissue.

2.3. Biochemical analyses

All blood samples were subjected to serum preparation by centrifugation at 10.000 g and 20 °C for 5 min (Microfuge 22R Centrifuge, Beckmann Coulter, USA). The serum was immediately stored at - 80 °C. The following serum parameters were analysed at the Core Unit for Biomedical Research at the Medical University of Vienna (using a Hitachi C311, Roche, Switzerland): blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides.

2.4. Assessment of atherosclerotic plaque area

The aorta was removed from its arch to its bifurcation, cleaned from perivascular tissue, washed (phosphate buffered saline without calcium and magnesium, 20 µM butylated hydroxytoluene, and 2 µM ethylenediaminetetraacetic acid, pH = 7.4), opened longitudinally, pinned on a black silicone plate with acupuncture needles (Standard Nr. 2, 0.20×15 mm, Asiamed, Germany) and fixed overnight in a formal-sucrose solution (containing phosphate buffer saline with 4% paraformaldehyde, 5% sucrose, 20 µM ethylenediaminetetraacetic acid). The fixed aortas were then incubated with Sudan IV (0.5% Sudan IV, 35% ethanol, 50% acetone) for 15 min to stain atherosclerotic plagues. Destaining of aortas was performed with 75% ethanol for 5 min. Pictures were taken with a single-lens reflex camera (camera: Canon EOS 550D, lens: Canon EF-S, f: 18-55 mm, IS, shooting mode: full automatic mode, Japan). Atherosclerotic plaque area was analysed using Adobe Photoshop CS4 for Windows (Adobe Systems, USA). Plaque area was calculated as percentage of either whole aortic area or segment area.

2.5. Intraperitoneal glucose tolerance test

IPGTTs were performed in five randomly picked mice per group after an overnight fast. Under anaesthesia the tail end was cut off and blood glucose levels were measured using a Contour TS (Bayer, Germany) before, and 15 min, 30 min, 60 min, 90 min, and 120 min after intraperitoneal injection of 1 g glucose/kg (Braun, Germany) bodyweight.

2.6. HMG-CoA-reductase activity assay

The activity of HMGCR was measured in the presence of vehicle control, 0.5 μ M leoligin, 5 μ M leoligin, or 50 μ M leoligin with a commercial kit following the instructions of the manufacturer (Sigma-Aldrich, USA).

2.7. Peroxisome proliferative-activated receptor-gamma activity assay

The ability of leoligin to interfere with PPAR- γ was measured in the presence of vehicle control, 10 µM, 50 µM, and 100 µM leoligin and with the addition of the well described agonist rosiglitazone (supplied with the assay kit indicated below, Indigo Biosciences, State College, USA) and antagonist GW9662 (Sigma-Aldrich M6191-5MG). A commercially available kit (Indigo Biosciences, State College, USA) was used to determine PPAR- γ activity according the manufacturers` instructions.

2.8. In silico modelling (HMG-CoA-reductase)

A ligand-based pharmacophore model for HMGCR inhibitors was developed using the bioactive conformations of known inhibitors as available in the Protein Data Bank (PDB) [19,20]. The 3D coordinates of atorvastatin (PDB code 1hwk), mevastatin (1hw8), simvastatin (1hw9), fluvastatin (1hwi), cerivastatin (1hwj), and rosuvastatin (1hw1) were aligned according to their positions within the HMGCR active site using the reference point-based alignment implemented in LigandScout 3.12 [21,22]. All statins mentioned above were also compiled into a 3D multiconformational "statin database". This database contained a maximum of 500 conformations per molecule and was calculated using OMEGA (OMEGA, version 2.3.3; OpenEye Scientific Software, Inc.: Santa Fe, NM, USA, 2009–2014) BEST settings as implemented in LigandScout [23,24].

To evaluate binding orientations for leoligin within the HMGCR active site, docking studies were performed with GOLD using a genetic algorithm for generating low-energy binding orientations. For docking, the PDB entry 1hw9 (chain A) was chosen because of its good resolution and the co-crystallized ligand simvastatin, which has a similar size as leoligin. The binding site was defined as an 11 Å sphere centred on Lys691. To ensure an optimal binding orientation, two hydrogen bond constraints were set: one with Lys691 and the other one with Ser684. In addition, Arg590 was set flexible. Goldscore was used as a scoring function. The program was set to define the atom types of the protein and ligand. The docking settings were validated by re-docking simvastatin into the crystal structure to ensure that the program is able to reproduce the binding orientation of the co-crystallized ligand.

2.9. In silico modelling (PPAR- γ)

Docking experiments were performed using AutoDock Vina 1.1 as implemented in LigandScout 4.09, using default settings. For docking, the PDB entry 2xkw (human PPAR- γ in complex with pioglitazone) [25] was selected. As positive control, the co-crystallized ligand pioglitazone was docked into the binding site without prior information on the ligand geometry or its orientation within the ligand binding domain. Out of the nine generated pioglitazone poses, the three most similar ones had RMSD values between 0.701 and 1.169 in comparison to the co-crystallized structure. This confirmed that the program settings were suitable to reproduce the correct pioglitazone binding geometry and its experimentally determined orientation within the binding site. For the prediction of the binding pose of leoligin, the same workflow was followed as for pioglitazone.

2.10. Immunofluorescence staining of HMG-CoA-reductase on liver sections

Immunofluorescence staining was performed on 5 µm liver paraffin sections. Liver tissue sections were subjected to standard deparaffinization technique followed by heat-mediated antigen retrieval with citrate buffer (pH = 6). Permeabilisation was achieved using 0.2% Triton X-100 in tris-buffered saline (TBS) for 5 min. Blocking of unspecific binding sites was performed using 1% bovine serum albumin (BSA) and 10% goat serum in TBS for 30 min. Slides were incubated with the primary antibody (anti-HMGCR antibody, NBP1-91996, Novus Biologicals, USA) overnight. Thereafter, liver tissue sections were incubated with the secondary antibody for 1 h in the dark, using Alexa Flour 488 goat anti-rabbit antibody (A11034, Life Technologies, USA). After washing in TBS with 0.1% Tween-20 (TBST), cell nuclei were stained with 5% propidium-iodide (PI) in TBS for 1.5 min. Slides were then mounted in ProLong Gold antifade reagent (P36930, Life Technologies, USA). Image acquisition was conducted using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software 2008 (Zeiss, Germany). Quantification of HMGCR area was performed using Adobe Photoshop CS4 for Windows (Adobe System, USA). HMGCR positive area was calculated in pixel and then related to the number of cell nuclei.

2.11. Hepatic protein extraction, Western blotting and immunodetection

For protein extraction, frozen liver samples of each mouse were cut in small pieces on ice and then suspended in RIPA-buffer (containing 50 mM TrisCl, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors). Samples were sonicated (Ultrasonic Processor UP200St, Hielscher, Germany) and the suspension was centrifuged at 10.000 rpm for 10 min at 4 °C (Microfuge 22R Centrifuge, Beckmann Coulter, USA). After separation of insoluble parts, protein concentrations were determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (23225, Thermo Scientific Fisher, USA) according to the manufacturers`instructions. Thereafter proteins were separated on a 10% sodium dodecyl sulphatepolyacrylamide gel (SDS-PAGE) and blotted onto nitrocellulose membranes. Subsequent to blocking of unspecific binding sites with phosphate buffered saline without calcium and magnesium (PBS -/-) containing 3% non-fat diet milk powder and 0.05% Tween-20, immunostaining was performed for ATP-binding cassette transporter A1 (ABCA-1) (mouse anti-ABCA-1 antibody; ab18180, Abcam, USA), scavenger receptor class B member 1 (SR-B1) (rabbit anti-SR-B1 antibody; NB400-104H, Novus Biologicals, USA), and LDL-receptor (LDL-R) (rabbit anti-LDL-R antibody; 3939-100, BioVision Inc., USA). Following 3 washing steps in PBS -/- with 0.05% Tween-20, membranes were incubated with either horse anti-mouse horseradish peroxidase (HRP)-linked antibody (7076, Cell Signaling Technology, USA) or goat anti-rabbit HRPlinked antibody (32460, Thermo Fisher Scientific, USA). After 3 washing steps, chemiluminescent detection was performed using either Super Signal West Femto Maximum Sensitivity Substrate (34096, Thermo Fisher Scientific, USA) or Super Signal West Pico Chemiluminescent Substrate (34011, Thermo Fisher Scientific, USA), followed by exposure of X-ray films (34091, Thermo Fisher Scientific, USA). Densitometric analysis of Western blot images was performed using ImageJ software.

2.12. Oil Red O staining of frozen liver sections and quantification

Liver sections were cut frozen (5 µm), air dried and then fixed in icecold formalin (7.5%; SAV-LP, Germany) followed by multiple washing steps with distilled water (B. Braun, Germany). Thereafter sections were air dried for approximately 1 h, incubated in pre-warmed (60 °C) propylene glycol (100%; Sigma-Aldrich, USA) for 3 min and then stained with a pre-warmed (60 °C) Oil Red O solution (0.5 g Oil Red O; Sigma-Aldrich, USA in 100 ml propylene glycol) for 60 min at 60 °C. Subsequently sections were differentiated with propylene glycol (85%) for 5 min, washed with distilled water twice for 3 min and then cell nuclei were stained with Mayer's Haematoxylin (Sigma-Aldrich, USA) for 1 min. Finally, sections were washed under running tap water for 6 min, then 3 times for 5 min with distilled water, mounted with Aquatex (Merck, Germany), air dried for 30 min and sealed with Entelan (Merck, Germany). Consequently, images were taken with Axiocam (Zeiss, Germany) and quantification of Oil Red O positive area was conducted using Adobe Photoshop CS4 for Windows (Adobe Systems, USA). Therefore, Oil Red O positive area was calculated in pixel in relation to total picture pixels.

2.13. Statistical analyses

Data was initially analysed for Gaussian distribution using Kolmogorov-Smirnov test with Lilliefors correction. Differences between the two groups were compared by either non-parametric Mann–Whitney *U* test or unpaired Student's *t*-test as applicable depending on the distribution of the tested variables. Two-sided ANOVA for comparison of multiple treatment groups was used. A value of p < 0.05 was considered significant. Statistical analysis was performed with IBM SPSS Statistics 20.0 for Windows (SPSS Inc., USA).

3. Results

3.1. Leoligin reduces cholesterol levels (in vivo) in ApoE -/- mice

Leoligin has previously been shown to interact both, in vitro and in vivo, with CETP by our group [16]. In order to test for the CETP-specificity of this cholesterol-lowering effect, we analysed the effect of leoligin in ApoE -/- mice, which do not express CETP, and surprisingly found a significant reduction of cholesterol levels in these mice. After 2 weeks of treatment, mice exposed to 1 µM, 10 µM, and 100 µM leoligin had



Fig. 1. Leoligin reduces cholesterol levels in ApoE -/- mice. Cholesterol levels were measured after 2 weeks (A[§], B[§]; n = 4 per group), after 5 weeks (C, D; control: n = 7, Leoligin 1 µM: n = 9, Leoligin 10 μ M: n = 7, Leoligin 50 μ M: n = 8), and after 16 weeks (E, F; control: n = 9, Leoligin 1 μ M: n = 9, Leoligin 10 μ M: n = 9, Leoligin 50 μ M: n = 7) of treatment with leoligin or vehicle control, respectively. (A, C, E) show the total cholesterol, LDL-cholesterol and HDL-cholesterol, whereas (B, D, F) depict the total cholesterol/HDL-cholesterol ratio and the LDLcholesterol/HDL-cholesterol ratio in the different treatment groups. Level of significance: *p < 0.05, **p < 0.01, and ***p < 0.001. [§]The two week serum samples originate from a separate experiment which included a 100 µM group instead of a 50 µM group.

significantly lower total serum cholesterol (p < 0.001, p = 0.001, and p = 0.028 respectively) and LDL-cholesterol (p < 0.001, p < 0.001, and p = 0.014 respectively) levels compared to the vehicle control group (Fig. 1A). Moreover, the total cholesterol/HDL cholesterol and the LDLcholesterol/HDL-cholesterol quotients were significantly lower in the 1 μ M and 10 μ M leoligin treatment group (p < 0.001 and p = 0.008, respectively) when compared to the vehicle control group (Fig. 1B). In order to test the duration of the cholesterol lowering effects of leoligin, we also analysed lipid levels at a later time point. After 5 weeks of treatment, total cholesterol and LDL-cholesterol remained significantly reduced in ApoE -/- mice treated with 1 μ M leoligin (p = 0.023 and p = 0.012 respectively), whereas there was an increase in HDL-cholesterol levels in the 50 μ M leoligin group (p = 0.028) resulting in a

2 weeks of treatment

Table 1

Effect of leoligin on serum triglyceride levels in ApoE-/- mice after 5 and 16 weeks of treatment.

		Control	Leoligin 1 µM	Leoligin 10 µM	Leoligin 50 µM
5 weeks	mean (±SD) p-value	40.57 (±5.71)	38.78 (±8.3) 0.634	36.57 (±10.45) 0.329	38.38 (±10.31) 0.626
16 weeks	mean (±SD) p-value	34.78 (±11.37)	29.11 (±7.17) 0.224	33.22 (±7.9) 0.74	24.29 (±7.34) 0.053

SD = standard deviation.

favourable LDL-/HDL-cholesterol ratio (Fig. 1C and D). Of note, after 2 and 5 weeks of treatment a clear dose-effect relationship between leoligin concentration and cholesterol ratios was observable as higher leoligin concentrations resulted in improved LDL-/HDL-cholesterol ratios. After 16 weeks of treatment we observed a significant increase in the LDL-/HDL-cholesterol ratio in the 10 μ M leoligin group (p = 0.031), but no significant differences in total serum cholesterol levels, LDL-cholesterol levels, and HDL-cholesterol levels (Fig. 1E and F).

Serum triglyceride levels were assessed after 5 and 16 weeks of treatment and did not show any significant alterations between the different treatment groups (Table 1). To exclude any toxic effects of leoligin in ApoE -/- mice, we analysed BUN, AST, ALT, LDH, and CK, but found no significant increases in leoligin-treated mice compared to controls after 16 weeks of treatment (Table 2).

3.2. Leoligin inhibits 3-hydroxy-3-methylglutaryl-CoA-reductase

To investigate the mechanism by which leoligin might alter cholesterol levels in our animal model of ApoE -/- mice, we analysed its effect on the activity of HMGCR *in vitro*. Surprisingly, 0.5 μ M, 5 μ M, and 50 μ M leoligin significantly reduced HMGCR activity in a cell free biochemical assay. 5 μ M and 50 μ M were able to inhibit HMGCR to a similar extent as a 100 μ M (final concentration) pravastatin solution (Fig. 2A).

3.3. Leoligin shares structural features with known HMG-CoA reductase inhibitors

In order to test for a potential statin-like binding and inhibition of HMGCR by leoligin, a molecular modelling workflow was employed. Bioactive conformations of statins co-crystallized within the HMGCR active site were aligned (Fig. 2B) and investigated for common protein-ligand interactions with the enzyme. The alignment of the bioactive conformations allowed for a positioning of pharmacophore features representing characteristic protein-ligand interactions: Three hydrophobic groups indicated positions for lipophilic protein-ligand contacts and four hydrogen bond acceptors represented the hydroxyl and carboxylic acidic groups on the opened lactone moiety (Fig. 2C).

For the leoligin mapping, a set of 3D conformations was calculated. Due to the chemical structure of leoligin, it was unlikely that the compound would map the statin model as it was. Particularly the four hydrogen bond acceptor features would be a challenge to map any compound, which has a chemical structure different from the statin class. We therefore allowed leoligin to leave out any two out of the four hydrogen bond acceptor features during the fitting. After having done so, leoligin mapped well into the model, which suggests that leoligin is able to form similar protein-ligand interactions with HMGCR as statins do (Fig. 2D).

3.4. Molecular modelling suggests a unique binding mode of leoligin to HMG-CoA-reductase

A binding mode of leoligin within the HMGCR active site was suggested by molecular docking. Leoligin showed binding interactions comparable to the ligand-based pharmacophore model and to the binding interactions of simvastatin. It shared a similar hydrogen bond network as simvastatin, and its hydrophobic side chains occupied the hydrophobic areas of the binding site. Whereas the statins form ionic interactions with Arg590 (Fig. 2E), leoligin established hydrogen bonds with this amino acid (Fig. 2F).

3.5. Effect of leoligin on atherosclerotic endpoints in ApoE -/- mice

In the next step we set to determine whether the beneficial alteration of lipid profiles by leoligin in ApoE - / - mice would result in reduced atherosclerosis in our model system. Therefore, we explanted the aortas from the aortic arch to the bifurcation after 16 weeks of treatment, stained the atherosclerotic plaques with Sudan IV and calculated the atherosclerotic plaque area as percentage of the whole aorta or segments of the aorta; i.e. ascending aorta plus aortic arch, descending aorta plus abdominal aorta, aortic bifurcation area. In our experimental setting, there was no significant effect of a systemic leoligin application on atherosclerotic plaque area (Fig. 3A and B). Further, we also analysed plaque morphology (plaque structure, cap thickness) in order to reveal potential alterations in plaque stability. To do so, aortic arch specimens were stained for alpha-smooth muscle actin (smooth muscle cells), von-Willebrand Factor (endothelial cells), and cell nuclei using TO-PRO-3. Image analyses of the samples and comparison between groups did not reveal significant differences between the leoligin groups and the control (for details see Online Supplementary Information and Supplementary Fig. S1A and S1B).

3.6. Long-term treatment with leoligin increases hepatic HMG-CoA-reductase expression in ApoE -/- mice

As the cholesterol-lowering effect of leoligin had disappeared after 16 weeks of treatment and no effect of leoligin on plaque area and

Table 2

Biochemical parameters in ApoE-/- mice after 16 weeks of leoligin treatment.

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		Control	Leoligin 1 µM	Leoligin 10 µM	Leoligin 50 µM
BUN (mg/dl)	mean (±SD) p-value	14.33 (±2)	16.44 (±2.24) 0.077	15.56 (±1.67) 0.178	19.43 (±8.14) 0.252
AST (U/l)	mean (±SD) p-value	35.89 (±5.04)	29.44 (±3.47) 0.006	66.56 (±46.41) 0.796	29.71 (±9.74) 0.121
ALT (U/I)	mean (±SD) p-value	18.22 (±7.92)	15.56 (±3.54) 0.489	28.44 (±15.77) 0.108	17.57 (±7) 0.758
LDH (U/l)	mean (±SD) p-value	235.11 (±82.87)	255.33 (±117.04) 0.678	419.67 (±317.42) 0.222	248.29 (±84.04) 0.758
CK (U/l)	mean (±SD) p-value	77.44 (±19.77)	84.78 (±29.13) 0.541	136 (±139.06) 0.222	62.43 (±15.7) 0.123

SD = standard deviation, BUN = blood urea nitrogen, AST = aspartate transaminase, ALT = alanine transaminase, LDH = lactate dehydrogenase, CK = creatine kinase.



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Fig. 2. Leoligin inhibits 3-hydroxy-3-methylglutaryl-CoA-reductase and molecular modelling and docking of leoligin with HMG-CoA-reductase suggests an unconventional binding mode. (A) HMGCR activity is shown as percent of the activity control (activity control equals 100%). Leoligin significantly inhibits the activity of HMGCR to a similar extent as 100 μ M pravastatin *in vitro*. **p < 0.01, ***p < 0.001. n = 4 for each data point. (B) Alignment of bioactive conformations of statin molecules bound to HMGCR. (C) Chemical functionalities shared by different statins. (D) Leoligin mapped to the common feature statin model. All but two chemical features (red spheres) were mapped by the ligand, suggesting similar protein-ligand interaction profiles compared to the currently used statins. (E) Simvastatin bound to HMGCR in the PDB entry 1hw9. (F) The statin forms hydrophobic contacts, hydrogen bond acceptors and donors, and a charged interaction with Arg590. Suggested binding orientation of leoligin within the HMGCR active site. Similar to simvastatin, hydrophobic contacts and hydrogen bond networks also including Arg590 are formed. Interaction types are colour-coded: red - hydrogen bond acceptor, green - hydrogen bond donor, yellow - hydrophobic, red star - negative charge.

morphology could be observed, we tested for a potential hepatic origin for the loss of leoligin activity. Accordingly, we first performed HMGCR staining in mouse liver sections. After two weeks of treatment with leoligin no differences in hepatic HMGCR expression were observed compared to the vehicle control group (Fig. 4A and B). However, after 16 weeks of treatment, paralleled by the disappearance of the drug effect on lipid levels and in the absence of inhibition of plaque formation by leoligin, we could observe a significant increase in hepatic HMGCR expression by leoligin (1 μ M (p = 0.04) and 50 μ M (p = 0.031)) compared to the control (Fig. 4C and D).

Further, we performed an Oil Red O staining to determine hepatic lipid and cholesterol depositions and measured liver and whole body weight at the end of the experiment. Compared to the control group, Oil Red O positive area was significantly increased in the 1 µM leoligin group (Fig. 5A and B), indicating more lipid/cholesterol deposition in the liver. In addition, liver weight/body weight ratio was slightly but significantly increased in this group (Fig. 5C).

However, absolute liver weight did not differ significantly between the groups, and also relative liver/body weight ratio (apart from the above exception) was not affected by the treatment. Microscopic morphologic inspection of the liver parenchyma did not reveal general alterations in liver architecture.

3.7. Low concentrations of leoligin may slightly increase LDL-receptor activity

In order to test for potential alterations in liver cholesterol uptake and metabolism, Western blot analyses of liver tissue homogenate for ABCA-1, SR-B1, and LDL-R, as well as in vitro cholesterol uptake and efflux analyses were performed. The increased cholesterol deposition in the 1 µM leoligin group cannot be explained by increased uptake of HDL-cholesterol via SR-B1 since expression of this protein was not affected (Fig. 5D and Supplementary Fig. S3). Additionally, HDL function seemed preserved as shown by measurement of HDL mediated cholesterol efflux in leoligin treated J774 mouse macrophages (Supplementary Fig. S2B). This is in line with additional in vitro experiments where leoligin did not affect HDL-cholesterol uptake in HEK293 cells (Supplementary Fig. S2A). However, the observed increased lipid content in the liver might be explained by enhanced LDL-R activity. Western blot analvsis of liver specimen showed at least a trend towards an increased LDL-R expression in mice treated with 1 and 10 µM leoligin. (Fig. 5D and Supplementary Fig. S3).

3.8. Leoligin reduces peak blood glucose levels in ApoE -/- mice

Because changes of lipid levels may also be caused by disturbed glucose metabolism, we measured fasting glucose levels and glucose tolerance after 2 and 16 weeks of treatment with leoligin or vehicle control. We did not observe any significant differences in basal fasting glucose values between the different treatment groups at either time point. However, after 2 weeks of treatment, peak blood glucose levels 60 min after intraperitoneal injection of glucose were significantly lower in the 10 μ M and 50 μ M leoligin group compared to the control (p = 0.009 and p = 0.002, respectively) (Fig. 6A). The intraperitoneal glucose tolerance test was also performed after 16 weeks of treatment, showing only a non-significant trend for reduced serum glucose peak values under leoligin treatment (Fig. 6B).

3.9. Leoligin is a weak PPAR-γ agonist in vitro and interacts with the ligand binding domain of PPAR-γ in in silico molecular modelling

Since PPAR- γ agonists are known to increase insulin sensitivity without affecting fasting glucose, we hypothesized that leoligin might interact with PPAR- γ activity. Based on the results of the IPGTT, where mice treated with leoligin had significantly lower peak blood glucose levels after intraperitoneal injection of glucose, we tested leoligin in a cell-based PPAR- γ activity assay. The cell based assay showed, that leoligin slightly increases (not statistically significant) PPAR- γ activity when applied alone (Fig. 7A). In the presence of the PPAR- γ agonist (rosiglitazone), leoligin decreased rosiglitazone induced PPAR- γ activity significantly (Fig. 7B). On the contrary, when the PPAR- γ antagonist (GW9662) was added to the cells leoligin was, once again, capable of increasing PPAR- γ activity (non-significantly; p = 0.074) (Fig.7C).

To describe the interaction of leoligin with PPAR- γ more precisely, molecular modelling and docking experiments were performed. Leoligin was predicted to bind to the PPAR- γ ligand binding domain at a similar site that is described for pioglitazone (Fig. 7D). Leoligin forms two hydrogen bond acceptors with Cys285 and Ser342 (red arrows, Fig. 7E and F). Additionally, extensive hydrophobic contacts are established between leoligin and PPAR- γ (yellow spheres, Fig. 7E and F).



Fig. 3. Effect of leoligin on atherosclerotic plaque formation in ApoE -/- mice. (A) Atherosclerotic plaque area is given as percentage of the whole aorta (total), ascending aorta and aortic arch (asc. aorta + arch), descending aorta and abdominal aorta (desc. + abd. aorta) and aortic bifurcation (bifurcation) after 16 weeks of treatment with leoligin and vehicle control (control: n = 9, Leoligin 1 μ M: n = 9, Leoligin 10 μ M: n = 9, Leoligin 50 μ M: n = 7). (B) Aortas were stained with Sudan IV to detect atherosclerotic plaques. Representative images are shown.

3.10. Leoligin is not detectable in Apo E - / - mouse serum and liver

In order to test for a possible metabolisation of leoligin at later time point as a potential explanation for the loss of leoligin's cholesterol lowering activity after 16 weeks of treatment, we set to determine leoligin concentrations in all mouse serum samples and mouse liver tissue samples using a novel gas chromatography – mass spectrometry-based approach. Surprisingly, leoligin could not be detected in our samples, neither at an early nor a late time point. The lower limit of detection (LOD) for serum was 0.5 μ M and for liver 1 μ M – for details see Online Supplementary Information.

3.11. Leoligin reduces weight gain in ApoE –/ – mice

As part of our studies on the effects of leoligin in Apo E -/- mice, we also measured body weight of the mice throughout the project. There were no differences in whole body weight between the 1 μ M leoligin, 10 μ M leoligin and vehicle control group. However, the 50 μ M leoligin treated group gained significantly less body weight compared to the control group (3.66 ± 1.29 g vs. 5.13 ± 0.67 g; p = 0.01; Fig. 8A) over a time period of 16 weeks. To rule out a possible influence of reduced energy intake, we measured the nutrition intake for all mice over time. No significant difference between the groups and the control could be observed (see Supplementary Fig. S4A).

4. Discussion

Leoligin, a compound isolated from the roots of the alpine plant Edelweiss, was shown to have beneficial effects on venous bypass graft patency as well as on serum cholesterol levels by interfering with CETP activity [16,17]. Dyslipidaemia is known as a major risk factor and driving force of atherosclerosis and subsequent CAD-development. To diminish the risk of development (primary prevention) and especially to reduce complications when clinically manifest (secondary prevention), CAD patients essentially rely on dyslipidaemia treatment. Among the treatment options such as diet changes and physical exercise, HMGCR inhibitors, statins in particular, are the major players in treating high total- and LDL-cholesterol serum levels and have become an indispensable part of CAD-management in the recent past [26]. Despite their huge clinical importance we have to deal on the one hand with serious adverse side effects (e.g. effects on skeletal muscle such as myositis or rhabdomyolysis [27] or a potential risk of developing new-onset diabetes mellitus [28]) and on the other hand with the non-achievement of recommended cholesterol-lowering goals with statins only [9]. Hence, the search for new lipid lowering agents is still a highly relevant task.

In this study, we identified leoligin as a potential new cholesterollowering drug that significantly reduces total serum cholesterol as well as LDL-cholesterol and leads to favourable cholesterol ratios in ApoE-/- mice via the inhibition of HMGCR. Although conflicting data on the effects of traditional HMGCR inhibitors in ApoE-/mice exist- (i) statins were shown to have no effect, an increasing



Fig. 4. Leoligin upregulates hepatic HMG-CoA-reductase expression after 16 weeks of treatment in ApoE -/- mice. (A) Hepatic HMGCR expression is given as positive area per hepatocyte after 2 weeks of treatment with leoligin and vehicle control (n = 4 per group)[§]. 5 images per mouse were evaluated. (B) ApoE -/- mouse livers were stained with an anti-HMGCR antibody and an appropriate secondary antibody (green signal). Cell nuclei were stained with Pl (red signal). Representative images are shown. (C) Hepatic HMGCR expression is given as positive area per hepatocyte after 16 weeks of treatment with leoligin and vehicle control (control: n = 9, Leoligin 1 μ M: n = 9, Leoligin 10 μ M: n = 8, Leoligin 50 μ M: n = 7). 5 images per mouse were evaluated. (D) ApoE -/- mouse livers were stained with an anti-HMGCR antibody and an appropriate secondary antibody (green signal). Cell nuclei were stained with an anti-HMGCR antibody and an appropriate secondary antibody (green signal). Cell nuclei were stained with an anti-HMGCR antibody and an appropriate secondary antibody (green signal). Cell nuclei were stained with an anti-HMGCR antibody and an appropriate secondary antibody (green signal). Cell nuclei were stained with Pl (red signal). Representative images are shown. [§]The two week samples originate from a separate experiment which included a 100 μ M group instead of a 50 μ M group. ^{*}Indicates p < 0.05, ^{**}p < 0.01, and ^{***}p < 0.01.

effect, or a decreasing effect on cholesterol levels and ii) it was suggested that statins decrease atherosclerotic plaque formation independent of their impact on cholesterol levels [29–31] - we think that leoligin, which is not a statin and does not have a statin-like structure, decreases cholesterol levels through direct inhibition of HMGCR *in vivo*.

Fig. 5. Oil Red O staining of liver cryosections, their quantification, relative liver weight and expression of hepatic ABCA-1, SR-B1 and LDL-receptor. (A) ApoE -/- mouse liver cryosections were stained with Oil Red O to quantify intrahepatic lipids and esterified cholesterol depositions (red). Representative images are shown. (B) Hepatic lipid content is given as Oil Red O positive area in percentage of whole liver area. 5 images per mouse/liver were evaluated (control: n = 9, Leoligin 1 μ M: n = 9, Leoligin 10 μ M: n = 7). (C) As an additional parameter for liver fat accumulation, the relative liver weight (% liver weight of whole body weight) was measured and analysed (control: n = 8, Leoligin 10 μ M: n = 9, Leoligin 10 μ M: n = 7). (D) ApoE-/- mouse liver lysates were separated on an SDS-PAGE, blotted onto a nitrocellulose membrane and then stained with different antibodies. Representative blots are shown at 254 kDa (ABCA-1), 82 kDa (SR-B1), and 160 kDa (LDL-R). *p < 0.005.



Δ



IPGTT after 2 weeks of treatment

B IPGTT after 16 weeks of treatment



Fig. 6. Leoligin reduces blood glucose peak levels in ApoE -/- mice. Intraperitoneal glucose tolerance test was performed (A) after 2 weeks of treatment and (B) after 16 weeks of treatment in ApoE -/- mice treated with leoligin and vehicle control (n = 5 per group), respectively. *p < 0.05, **p < 0.01.

In silico docking and modelling studies suggest that binding of leoligin to HMGCR may be unconventional. In detail, common feature pharmacophore model fitting and the docking show that leoligin competitively occupies the active site of HMGCR similar to the currently used statins. However, although it binds at a similar location it does not establish the same interaction pattern as statins. Typically, statins contain an opened lactone ring and can therefore form an ionic interaction with Arg590 in the binding site. Due to the lack of an acidic moiety in leoligin, this charged interaction is replaced by hydrogen bonds between two methoxy groups of leoligin and Arg590. According to the proposed binding mode there is still space adjacent to the leoligin molecule after locking into its binding site. Therefore, hydrophobic substitution of leoligin's ester side chain may increase its activity against HMGCR.

In vivo the cholesterol-lowering effects of leoligin were found after 2 and 5 weeks of treatment, but not after 16 weeks. Endo et al. previously described a marked and compensatory hepatic induction of HMGCR expression in mice under statin treatment [32]. Our immunofluorescent analyses of liver tissue also showed a strong upregulation of HMGCR after 16 weeks of treatment in the leoligin groups, resulting in a significantly increased hepatic lipid/cholesterol deposition in the 1 μ M leoligin group. This hepatic counterregulation could explain the lack of an enduring leoligin effect on cholesterol levels. Moreover, we observed a constant decrease in serum cholesterol levels in the control group over time, which may mask the cholesterol-lowering effects of leoligin. Despite the beneficial effects of leoligin described above, histological analyses of atherosclerotic plaque area and plaque structure/composition showed no protective effect. Based on this discrepancy we hypothesize that the increased hepatic HMGCR expression resulting in unchanged cholesterol levels at the endpoint may have overruled potential anti-atherogenic effects of leoligin. Importantly however, we need to reinforce that the relationship between pharmaceutical targeting of the HMGCR with its subsequent influence on cholesterol levels and its effects on atherosclerotic plaque formation in ApoE -/- mice remains controversial [29].

Our results showed that leoligin potently decreases serum glucose peaks after intraperitoneal glucose injection in ApoE -/- mice without influencing fasting glucose. Elevated postprandial glucose peaks have been identified to be a risk factor for CVDs (even in the absence of elevated fasting glucose), also in non-diabetic patients. There is evidence that aggressive treatment of postprandial hyperglycaemia may be necessary to reduce this risk [33]. To elucidate the mechanisms of action by which leoligin decreases peak glucose levels, we investigated a potential interaction with PPAR- γ activity, a known target of the anti-diabetic glitazones as reviewed by Ahmadian et al. [34]. In a cell-based in vitro assay, leoligin was able to increase PPAR- γ activity in control cells and also in cells treated with the PPAR- γ antagonist GW9662. On the contrary, leoligin decreased rosiglitazone induced PPAR- γ activity. Based on these results, we suggest that leoligin is a weak PPAR- γ agonist, which could explain its effects on blood peaks glucose levels. Further we suggest that leoligin, when combined with the strong agonist rosiglitazone – by competition for the PPAR- γ binding sites – reduces rosiglitazone induced PPAR-γ activity. Further, in silico docking and modelling and most of all biochemical assays are needed to precisely define the observed phenomena. Interestingly, the peak glucose reducing effects of leoligin, similar to the loss of cholesterol lowering activity, also vanished after 16 weeks. Again, a possible counterregulatory effect as demonstrated for hepatic HMGCR might be at play. Nevertheless, a common and central process such as drug metabolisation may lead to the loss of activity of leoligin in both phenomena. As leoligin was not detectable by GC-MS analyses in mouse serum (LOD 0.5 µM) or liver samples (LOD 1 µM), the question whether differing enzymatic degradation of leoligin is relevant for the ceasing activity after 16 weeks has yet to be answered. Regarding the reduction of serum cholesterol levels we provided data that concentration as low as 0.5 µM leoligin still potently inhibits HMGCR enzyme activity.

In our previous study using oral leoligin application and CETP expressing mice [16], leoligin could be detected in the circulation, clearly showing the transport of leoligin from the digestive tract into the circulation. Given the fact that CETP is a blood circulating protein which binds leoligin [16], it is assumed that CETP, which is not expressed in wild type and ApoE -/- mice, might stabilize leoligin in, or reduce clearance from the circulation through the digestive tract and is present in the serum of ApoE -/- mice, but may be - by binding to the intracellular HMGCR - "cleared" from the circulation. Importantly, other mechanisms by which leoligin interferes with serum lipid levels may be at play. The assessment of *in vivo* cholesterol metabolism (by radioactive labelling of cholesterol) combined with the study of a potential inhibition of cholesterol uptake in the gut may shed light on these potentially additional mechanisms.

Another effect of leoligin that could be observed – but not explained – in the course of the present project was that in the 50 μ M leoligin group treatment resulted in reduced weight gain of animals. In order to exclude reduced food consumption by the 50 μ M leoligin group animals, food consumption per animal was analysed and found to not differ compared to the other groups. Further we analysed a significant number of parameters (*i.e.* BUN, AST, ALT, LDH, CK), which indicate organ toxicity. None of these parameters was increased in leoligin treated animals, suggesting that leoligin is not toxic to Apo E -/- mice when applied orally over 16 weeks and up to 50 μ M.



Fig. 7. Leoligin is a weak PPAR- γ agonist *in vitro* and molecular modelling and docking of leoligin to PPAR- γ suggests a similar binding mode as described for pioglitazone. Leoligin (A) slightly increases PPAR- γ activity when applied alone, (B) significantly decreases PPAR- γ activity when combined with a PPAR- γ agonist (rosiglitazone) and (C) reverses PPAR- γ antagonism (antagonist used: GW9662). *p < 0.05. (D) The three most similar predicted binding poses of leoligin (cyan) superimposed on the X-ray structure of pioglitazone (grey) bound to the PPAR- γ ligand binding domain (pocket). (E) and (F) 2D and 3D predicted protein-ligand interactions between leoligin and the PPAR- γ ligand binding domain. The ligand forms two hydrogen bond acceptors with Cys285 and Ser342, respectively (red arrows). Additionally, extensive hydrophobic contacts are established (yellow spheres).

With the positive effects of leoligin on serum cholesterol, peak glucose levels, and weight gain reported in this study, as well as with its intimal hyperplasia inhibiting properties and its CETP-modulating activity, we think that leoligin possesses anti-atherogenic characteristics which are of high interest. Since dyslipidaemia, hyperglycaemia, and obesity are major risk factors in the pathogenesis of atherosclerosis and CVD, leoligin may be a novel and potential therapeutic agent for primary and secondary prevention. Without doubt, many more studies and analyses are needed prior to the first study in human.

Author contributions

All authors reviewed the manuscript and agreed to submission. B.S. and D.B. designed the project. B.S., B.M., G.L., M.G., H.S., P.E., A.R., and D.B. planned the experimental design. B.S., B.M., A.T., D.S., A.V., F.P., K.H., K.A., H.O., and A.R. planned and conducted the experiments. B.S. wrote the manuscript. B.S., B.M., A.T., D.S., A.V., G.L., M.G., H.S., P.E., A.R., and D.B. discussed the data.

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Fig. 8. High-dose leoligin treatment decreases weight gain in ApoE-/- mice. (A) Weight gain is given in gram over the whole treatment period (16 weeks). Mice were weighted once every week to evaluate their weight gain (control: n = 9, Leoligin 1 μ M: n = 9, Leoligin 10 μ M: n = 9, Leoligin 50 μ M: n = 7).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.yjmcc.2016.08.003.

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